The Quarterly Journal of Microscopical Science

FOUNDED 1853

September 1954

VOL. 95 · PART 3 (THIRD SERIES, NO. 31)

Joint Editors

C. F. A. PANTIN, Sc.D., F.R.S.

JOHN R. BAKER, D.Sc.

Assisted by

WILLIAM HOLMES, D.Phil.

Published for the Company of Biologists Limited

OXFORD: AT THE CLARENDON PRESS · LONDON: GEOFFREY CUMBERLEGE

Price 30*s. net*

Subscription for Volume £4. 4*s.*

TO CONTRIBUTORS

Study of this notice may accelerate publication by three months.

Contributions to the Journal should be sent to Dr. C. F. A. Pantin, Dept. of Zoology, Downing St., Cambridge. If any preliminary communication on the subject of a paper offered to the Editors has been sent to another journal, a note to this effect must accompany the manuscript.

Manuscripts must be typewritten (double-spaced). Typescripts should be fully corrected. An allowance of 10*s.* per sheet of sixteen pages is made for alterations in proof, contributors being responsible for any excess.

Manuscripts should be as concise as possible. Papers that conform to the instructions given on this page and do not exceed 6,000 words will usually be published more quickly than others.

Extensive introductory matter and long discussions, commonly required in theses for university degrees, should **not** be included in the manuscript.

Words to be printed in italics should be underlined. Division of the matter into subheadings must follow a consistent plan and excessive subdivision must be avoided.

All manuscripts must be accompanied by a **summary,** not exceeding 5 per cent. of the length of the paper and in no case exceeding 500 words. The summary should contain no illustration or table and should be intelligible by itself without reference to illustrations or tables in the body of the paper. The summary must be a brief statement of the chief results obtained: it must not be a mere list of subjects discussed. It must be placed at the beginning of the paper, after the author's name and address.

Footnotes must not be used.

Acknowledgements must be placed at the end of the paper, before the references.

References should be given in the following style:

Goodrich, E. S., 1919. *The evolution of living organisms.* London (Jack).
Lankester, E. R., 1904. Quart. J. micr. Sci., **48,** 165.

If it is considered desirable to give the titles of papers, they should be placed in inverted commas immediately after the dates of the references.

ILLUSTRATIONS

These should not be redundant. Authors may be asked to provide a grant for excessive illustration. Illustrations (including lettering) should be arranged so that they may be printed as figures of a size appropriate to a page of the journal. The maximum size of a printed figure is 13 cm. × 20 cm. Text-figures should be made about half as large again as the size required in print. Thus a drawing destined to occupy a whole page should be about 19½ × 30 cm., including lettering. Very large illustrations (whether text-figures or plates) are not accepted by the Editors. Nothing should be sent that will not fit easily within an envelope measuring 16 × 12 inches (about 40 × 30 cm.).

All figures, both text-figures and plates, should be numbered in a single series (figs. 1, 2, 3, &c.). There must not be one numbered series of text-figures and another separately numbered series of plates. The Editors will decide which shall be reproduced as plates and which as text-figures. If several drawings or photographs are grouped in a single figure, they must be distinguished by capital letters (A, B, C, &c.). All photomicrographs must be carefully mounted on white cardboard. If several photomicrographs are to form a single plate or text-figure, they must be mounted in their proper places on a single piece of cardboard.

Drawings should be 'line and dot' in black process ink.

The lettering of figures should be written clearly on accurately imposed outlines on tracing-paper. Abbreviations should not be used unless this is absolutely necessary.

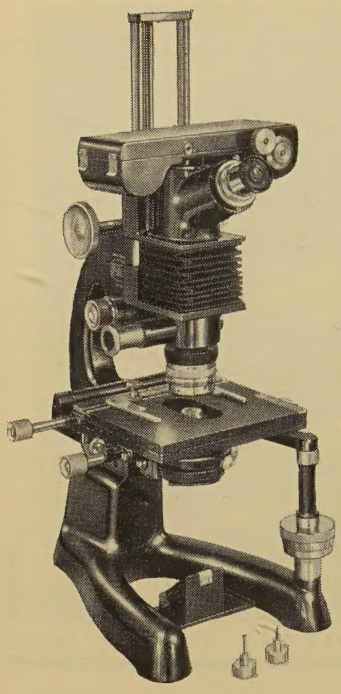
The magnification of a figure must be indicated by a scale drawn on it. It must not be stated as a number intended to be printed in the legend to the figure.

A typewritten (double-spaced) list of the legends of all figures must be provided.

Authors receive 50 copies of their contributions gratis, and may buy additional copies if they apply to the Editors when they return the corrected proofs.


COOKE
MICROSCOPES
M25
SERIES
Cooke Troughton & Simms LTD
YORK ENGLAND

[1 *front*]



BECK LONDON

REFLECTING MICROSCOPE

The Beck Reflecting Microscope has been designed to enable the full advantage to be taken of the new series of reflecting objectives, developed from the designs of the Wheatstone Laboratory and Medical Research Council Biophysics Research Unit at King's College, London.

They contain no lenses or correcting plates, having two reflecting surfaces only.

They are completely achromatic and transmit all radiations from vacuum ultra-violet to long wave-length infra-red. There is no spherical aberration. They are specially suitable for all forms of micro-spectrography.

Full particulars will be sent on application

R. & J. BECK LTD.

69/71 Mortimer St., London, W.1

The **BRITEX**

MICRO PROJECTOR

The Britex MICRO-PROJECTOR has been designed primarily as a visual aid to middle-school education. With this in view, a perfectly adequate precision instrument has been produced at a very reasonable cost.

FOUR MAIN USES:

1. **Direct projection on to a screen.**
2. **For drawing. By using mirror, image can be projected on to the bench.**
3. **For using liquids on stage. Projector can be swung into vertical position and the image projected on a screen via the mirror.**
4. **Visual use. The rod and mirror can be unscrewed and a filter (accessory) placed between light source and stage, making the Projector usable as an ordinary microscope.**

Costing £37. 10s. in its simplest form, the Projector is supplied with a single nose-piece, a 1 in. objective and a x6 or x10 eyepiece. Other accessories are available (as illustrated).

Available from all reputable instrument dealers.
Literature on request.

A PROJECTION HEAD for use with the Britex PROJECTOR is also available.

BRITEX (SCIENTIFIC INSTRUMENTS) LIMITED

Manufacturers of Microscopes, Magnifiers, Telescopes, &c.

523-4 BANK CHAMBERS, 329 HIGH HOLBORN, W.C.1

Telephone: HOLborn 7166 and 2090

The Use of the Interference Microscope to Determine Dry Mass In Living Cells and as a Quantitative Cytochemical Method

By H. G. DAVIES, M. H. F. WILKINS

(*Medical Research Council Biophysics Research Unit, King's College, Strand, London, W.C. 2*),

J. CHAYEN

(*Wheatstone Physics Laboratory, King's College, London, W.C. 2*),

AND

L. F. LA COUR

(*John Innes Horticultural Institute, Bayfordbury, Herts.*)

With 4 plates (figs. 7, 8, 9, and 11)

Summary

1. The total mass M of substances other than water (the dry mass) in the living cell can be obtained from the expression $M = \phi A/\chi$, where ϕ is the optical path difference (o.p.d.) due to the cell and A its projected area. The method makes use of the fact that the refractive increments α ($\chi = 100\alpha$) of most substances in cells are approximately the same, and independent of concentration. Values for χ have been tabulated.

Inaccuracies in the measurement of dry mass due to variations in χ (using λ average = 0·18) will be less than ± 10 per cent. in cells containing nucleic acids, proteins, and lipoproteins. When appreciable quantities of other substances are present the inaccuracy may be somewhat greater.

When the total dry mass of living cells is determined in a medium other than water (e.g. isotonic solution), a correction term involving the thickness must be determined; this correction is often small.

2. Optical path differences have been measured with the Dyson interference microscope, and different methods for doing this, together with the interpretation of the image in interference contrast, are discussed.

3. The total dry masses and, in some cases, the concentrations of dry substance in a variety of biological objects including *Amoebae*, pollen grains at various stages of development, nuclei of cells in tissue culture, and sperm heads have been determined.

In *Tradescantia bracteata*, during development from the microspore to the mature pollen grain, the dry mass increases by about tenfold. The dry masses of mature pollen grains were measured before and after successive digestion with ribonuclease, which removed about 4 to 14 per cent. of the dry mass, and with trypsin, after which about 40 per cent. of the original dry mass remained.

In living ram sperm heads the ratio of deoxyribose nucleic acid to total dry mass determined by ultra-violet and interference microscopy respectively is 40 per cent. This is in good agreement with the value 45 per cent. obtained by bulk biochemical methods.

4. The interference microscope has been used to measure the refractive index of cells and, hence, the concentrations of dry substances in them, by immersing them in media of different known refractive indices. The application of this method to fixed cells is discussed theoretically. In experiments on fixed ram sperm heads the expected linear relationship between o.p.d. and refractive index of the immersion medium was obtained. Data on the average concentration of dry substance in ram sperm heads, the localized refractive index, and concentration in the denatured submicroscopic particles

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 271-304, Sept. 1954.]

in the head, the percentage of the head volume occupied by them, and the geometrical thickness of the head were obtained.

5. Factors affecting the accuracy of the measurements of o.p.d., such as glare in the microscope, light scatter or absorption by the object, &c., are outlined.

Contents

Introduction

THE aim of cytochemistry is to determine the quantity and distribution of chemical substances in living cells in various states, and thus ultimately to relate chemical composition to biological process and to interpret these processes in terms of chemical change. Compared with chemical methods cytochemical techniques are very limited, insensitive (in terms of concentration) and uncertain. A number of more or less specific methods exist for certain substances and some of these methods are more or less quantitative. Apart from

nprovement of these methods, what is especially needed in cytochemistry is e ability to measure the total amount of substance other than water in a cell. easurement of this 'dry mass' of cells is necessary if one wishes to express uantities of particular substances in cells as fractions of the whole and as an d in relating the quantity of one substance to that of another. Such dry mass easurements are also of basic importance in so far as increase of total dry ass of a cell may be taken as a measure of its growth.

It has been shown (Davies and Wilkins, 1951, 1952; Barer, 1952) that easurement of cell refractivity or optical path length can be used to measure e mass of total substance other than water in a living cell or in any of its rts. This method makes use of the fact that the refractive increments of most bstances in cells are approximately the same and independent of composi-on. The measurements may be made quite readily with the interference icroscope and the method may be useful in many fields of research. The ethod, being optical, has the advantage that it may be used on living cells, us avoiding fixation artifacts. It is possible to follow changes in any one living ll since the radiation used does not damage the cell. The method of Eng-röm and Lindström (1949) has previously been the only method of measur-g dry masses of cells; it is only applicable to cells which are dried.

Some of the main applications of the methods are briefly as follows:

1. In general, the dry mass of a cell thus determined may, when measured ver an interval of time, be used as a measure of growth.

2. When cells are largely composed of nucleic acids and protein, the nucleic id content may be found by ultra-violet absorption and the protein by easuring total dry mass and subtracting the mass of nucleic acid.

3. If the cells are fixed and extracted with chemical reagents, or digested ith enzymes, the amount of substance removed may be measured directly.

4. Morphology has been concerned with the linear dimensions of cells. It ill now be possible to obtain quantitative data on the masses and concentra-ons of substances other than water in chromosomes, nucleoli, fibrils, &c.

5. Determination of concentration gradients in cells can be made (e.g. those sociated with cellular or embryonic differentiation).

The interference microscope method can give dry masses with a satisfactory egree of accuracy if the object studied has suitable properties. A great many ological objects will be suitable, but it must be stressed that, as in micro-ectrography, choice of unsuitable objects may give rise to serious errors of easurement.

Relationship between Optical Path Difference and Dry Mass

If m is the mass in grams of material other than water in a homogeneous ject of area A cm.^{2} and thickness t cm. situated in water, then

$$m = \frac{\phi_w A}{\chi}, \qquad (1)$$

here ϕ_w is the optical path difference (o.p.d.) produced by the object, that is,

the difference in the optical paths through the object and an equal thickness
water; ϕ_w is measured by means of an interference microscope in terms
wavelength (*in vacuo*) of the light used. Thus

$$\phi_w = (\mu_0 - \mu_w)t \text{ cm.},$$

where μ_0 and μ_w are the refractive indexes of the object and water respectivel
χ for solutions is 100α, where α, the specific refractive increment, is given b

$$\alpha = \frac{\mu_0 - \mu_w}{c},$$

where μ_0 and μ_w are the refractive indexes of the solution and water respe
tively, and c is the concentration expressed as grams of dry material per 100 c.
of solution ($100\ m/At$). χ for a dry object is given by

$$\chi = \frac{\mu_0 - \mu_w}{\rho},$$

where μ_0 is now the refractive index of the object and ρ its density (m/A
Expression (1) is derived directly from expressions (2), (3), and the d
mass m may be determined by measuring ϕ_w and A if χ is known.

The accompanying table 1 shows that the values of χ for substances occu
ring in cells do not vary very much and, furthermore, the values of χ a
approximately independent of concentration. In cells containing mainly sol
tions or gels of protein (χ = about 0·19), lipoproteins (χ = about 0·17 for 75 p
cent. fat content), and nucleic acids (χ = about 0·20, 0·16→0·17), the err
in the dry mass determinations due to the assumption of an average χ val
of 0·18 will not exceed ±10 per cent. This error could arise only if the obje
consisted entirely of only one of these substances, and in a mixture the err
would be less. When appreciable quantities of carbohydrates (χ = abo
0·14), fats (χ = about 0·14), and salts (χ = about 0·12→0·21), are prese
the error will be greater, but often the composition of the object will be roug
known and this error can be reduced by selecting the value of χ according
The appropriate average value of χ may be determined from the expressio

$$\chi_{av} = x_1\chi_1 + x_2\chi_2 + \ldots,$$

where x_1, x_2,... are the fractional dry masses of each substance present a
χ_1, χ_2,... are the corresponding χ-values. In the results presented later we ha
not attempted to estimate the correct average χ-value used, but have used t
value 0·18.

With living cells it is often desirable to make measurements in media oth
than water alone, for example, in isotonic salt or sugar solutions. The opti
retardation ϕ_m is now measured relative to the medium of refractive index μ
so that

$$\phi_m = (\mu_0 - \mu_m)t,$$

where μ_0 is the refractive index of the object as before; that is, it is assum

hat no substance from the medium enters the cell or vice versa. By combining equations (3) and (6) the mass m of material other than water in the cell is iven by

$$m = \frac{A\phi_m}{\chi} + (\mu_m - \mu_w)\frac{At}{\chi}. \qquad (7)$$

Hence the correction term can be determined by measuring the refractive ndex of the medium and t, the cell thickness. If m' is the amount of material

TABLE I

Compound	*Physical state*	*Density*	*Refractive index*	χ
Ovalbumin[1]	1·61% sol.	..	..	0·187
Ovalbumin[1]	6·45% sol.	..	..	0·188
Gelatin[2]	Dry solid	1·27	1·525 av.	0·151
Tobacco mosaic virus	Dry solid	1·335 av.[4]	1·534[5]	0·151
Tobacco mosaic virus[3]	Dil. soln.	..	..	0·17
Sodium thymonucleate[6]	Dil. soln.	..	..	0·16
Sodium thymonucleate[7]	Dil. soln.	..	..	0·20
Ribose nucleic acid[8]	2% soln.	..	..	0·168
Fats[2]	Nat.	0·93	1·46 av.	0·14 av.
α-Lipoprotein[9]	Dil. soln.	..	..	0·17
Glycine[10]	Dil. soln.	..	..	0·179
Alanine[10]	Dil. soln.	..	..	0·171
Valine[10]	Dil. soln.	..	..	0·175
Tryptophane[10]	..	..	..	0·25
Sucrose[2]	2% soln.	1·006	1·3359	0·141
Sucrose[2]	Cryst.	1·588	1·558	0·141
Starch[2]	Solid	1·50	1·53	0·133
Sodium chloride[2]	5·25% soln.	1·035	1·342	0·163
Sodium chloride[2]	Cryst.	2·165	1·544	0·097
Potassium chloride[1]	10% soln.	1·07	1·3449	0·115
Potassium chloride[1]	Cryst.	1·98[4]	1·490	0·079
Calcium chloride[2]	17% soln.	1·143	1·374	0·21
Calcium chloride[2]	Cryst.	2·512	1·52	0·075

References cited: 1. Perlman and Longsworth (1948); 2. *Handbook of chemistry and physics*; . Oster (private communication); 4. Bawden and Pirie (1937); 5. Bernal and Fankuchen 1941); 6. Tennent and Villbrandt (1943); 7. Barer (1952); 8. Davies and Wilkins (unpublished); 9. Armstrong and others (1947); 10. Adair and Robinson (1930).

ther than water in a volume of the medium equal to that of the cell, then (cf. quation (3))

$$(\mu_m - \mu_w) = \frac{\chi' m'}{At}, \qquad (8)$$

where χ' is the value appropriate to the medium. It is sometimes more convenient to use another expression for the dry mass m of the object obtained rom (7) and (8); that is,

$$m = A\frac{\phi_m}{\chi} + \frac{\chi'}{\chi}m', \qquad (9)$$

where m' can be obtained from the concentration of the medium; χ' for Tyrode solution = about 0·16; χ' for sucrose solutions is 0·14.

It will be seen that the correction involved when dry masses of cells ar measured in media other than water requires a determination of cell thickness and this may be difficult to obtain. However, the correction term involving th thickness t is small when the concentration of material in the medium is small relative to that in the cell. This is often the case and then it is sufficient t make only a very rough estimation of cell thickness. In general, the optica retardation varies over the image of the cell and the total dry weight is deter mined by an appropriate integration over the cell projected area.

The Interference Microscope

The principles of the interference microscope are described, with referenc to the Dyson microscope (Dyson, 1950), which was mainly used in the experi mental work described later. The principle is similar to that of the Jamin

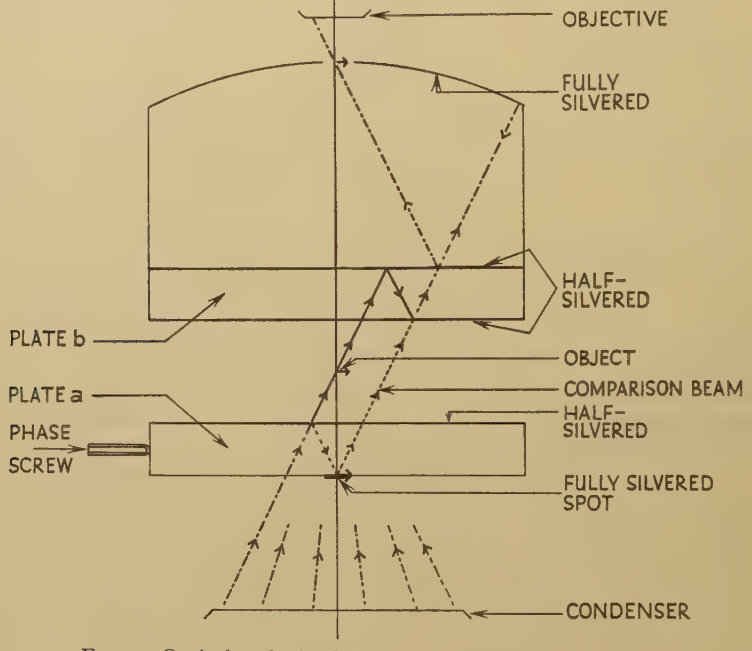


Fig. 1. Optical paths in the Dyson interference microscope.

interferometer. A glass plate (*a*, fig. 1), not quite plane parallel but with very small wedge-angle, is situated between the condenser and object, which is mounted in the usual way between glass slide and coverslip. The upper surface of the plate is half-silvered while the lower surface bears a small fully silvered central spot. Part of the illuminating cone traverses this plate (only single ray is shown in fig. 1) and illuminates the object (full line), while part (broken line) is reflected downwards and then upwards from the silvered spot so as to form a comparison beam which passes through an annular area around and much larger than, the region occupied by the object in the field of view o

the microscope. This annular region has internal and external radii of about 0·9 and 1·5 mm. respectively. The two beams are recombined by a second plate (*b*), situated above the object, identical with the first except that it is partially silvered on both faces. Part of the comparison beam now traverses this plate without reflexion, whilst part of the illuminating beam which has passed through the object undergoes a double reflexion. Two images are thus formed, situated near the fully silvered spot, one of the light source alone and the other an image of the light source with the object superimposed. When the plates are correctly aligned the two coherent images coincide and interference will occur

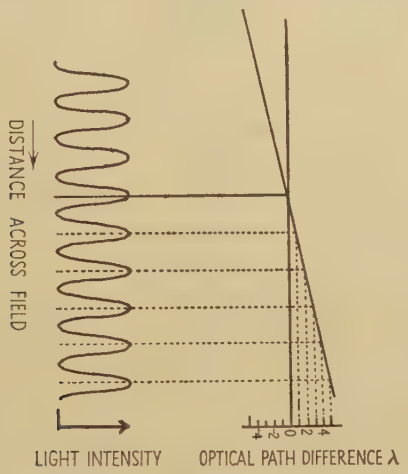


FIG. 2. The optical path difference between the two interfering beams varies linearly across the field of view, producing a light intensity distribution of the form shown in the lower curve.

(the phases of the light vibration in two coherent images bear a constant relation to each other). A glass block situated above plate *b*, with a silvered spherical upper surface, forms a real image which can then be viewed at a central transparent hole by an ordinary high-power objective (Dyson, 1949).

As a result of the wedge-like shape of the plates the optical path difference between the two beams varies linearly across the field. Hence, the field of view is, in general, seen to be crossed by a number of equally spaced straight interference bands (fig. 2). The light intensity across the field varies according to the expression $a+\cos\theta$ (derived from equation (15), where θ is the phase difference ($2\pi/\lambda \times$ o.p.d.) between the two interfering beams. The contrast has its maximum value, and the dark bands are perfectly black, when the two beams are equal, and then the constant a has the value unity. The separation of the bands, which is determined by the rate of variation of the path difference across the field, depends both on the wedge angle and the angle between the axes of the wedges. For a given wedge angle the band separation can be altered by rotating the upper plate (in its own plane) relative to the lower one, and is infinite (zero path difference variation over the field), and the field uniformly illuminated, when the wedge axes are parallel. When the wedge axes

are at 180° to each other the band spacing has its minimum value. By moving the lower plate in a direction normal to the optic axis and to the wedge axis the path difference between the two interfering fields can be continuously varied; such movement is produced by operation of the 'phase screw' and causes the band system to move as a whole across the field in a direction at right angles to the bands.

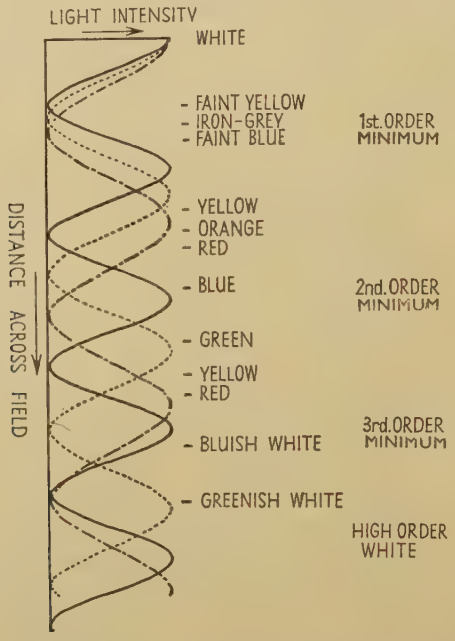


FIG. 3. Light intensity distribution across the field for three monochromatic wavelengths, blue ——, green - - - - - - -, red –·–·–·. In white light the colours are as indicated.

With white light the bands show Newton's colour sequence with a central white band, which indicates that the optical path difference between the two interfering beams is zero along that band. The way in which this white light band system arises is shown in fig. 3, where interference bands are drawn for three individual wavelengths. When the white light continuous spectrum is used the colours are roughly as indicated, and for large path differences overlap to produce a white light. These white light bands are used generally in interferometric methods in the manner indicated below.

INTERPRETATION OF IMAGE AND PRINCIPLES OF MEASUREMENT

Optical path differences are observed and measured with the interference microscope by methods which are basically the same as those commonly used with many interferometers (e.g. Jenkins and White, 1951; Williams 1948), but as many workers may not be well versed in interferometry a description of methods is given below at some length and in what we hope are simple terms.

The way the microscope can be used will be made clearer if we consider the ppearance of some simple objects of known geometrical form when they are ıtroduced into the field of view, first when the field is crossed by interference ands, and secondly when it is of uniform light intensity.

. *Background field crossed by interference bands*

The path difference between the two interfering beams in the microscope ıen varies linearly across the field in a direction at right angles to the bands nd is constant along any band. Hence each point in the field corresponds with certain optical path difference between the two light beams, and this varies y one wavelength from one band to the next. When light rays pass through n object in the field the difference of refractive index between the object and ıe surrounding medium causes one light wave to be accelerated or retarded. Ience the optical path difference between the beams at a point where the bject lies in the field is altered by an amount $(\mu_0-\mu_m)t$, where μ_0 and μ_m are ıe refractive indices of the object and the medium respectively, and t is the ıickness of the object.

(*a*) *Large uniform object.* If the object is a large homogeneous strip of uniform ıickness the optical path differences associated with all points in it are altered y the same amount. This causes the whole band system to be displaced ırough a distance D given by $(\mu_0-\mu_m)\,t\cdot d/\lambda$, where d is the band separation. Ience, by measuring the distances D and d the optical path difference due to ıe object can be determined as $\phi_m = (\mu_0-\mu_m)t = \frac{D}{d}\cdot\lambda$, where λ is the mono-

hromatic wavelength (*in vacuo*) of the light used.

The appearance of such a strip object is shown in fig. 4, A (upper portion), ith the variation in light intensity across the field and object shown as full nd broken curves respectively in fig. 4, B. When monochromatic light is used ll the interference bands look the same and hence it is not possible to tell hich band in the object band system corresponds with a given band in the ackground field. Thus, the band displacement, that is, the distance between orresponding points in the object and background field where the overall ptical path differences between the two beams are equal, may be d_1 (fig. 4, B), $_1\pm d$, $d_1\pm 2d$, &c. This ambiguity is resolved by using light of several wave-ngths, for example white light, and is achieved by measuring the separation between the white band of zero path difference seen in the background field id in the extended object. An alternative method is to set the white band ppearing in the background field on to a reference line which passes through ıe object. By operating the 'phase screw' the white band is brought to lie on ıe reference line in the object and a count is made of the number n of bands assing the line. Then the optical retardation $\phi = n\lambda$. This number n will in eneral consist of a whole number and a fractional part. The fractional part of like the fractional displacement d_1, can only be roughly estimated by using hite light bands.

The displacement D, measured above, may arise from an object of higher or

lower refractive index than the surrounding medium. Whether an unknown object advances or retards the phase of the light wave can only be determined by finding if the optical path difference is increasing or decreasing in a given direction across the field at right angles to the bands. This is done by viewing an object whose refractive index is, for instance, known to be higher than the surrounding medium and noting the direction in which the white central band

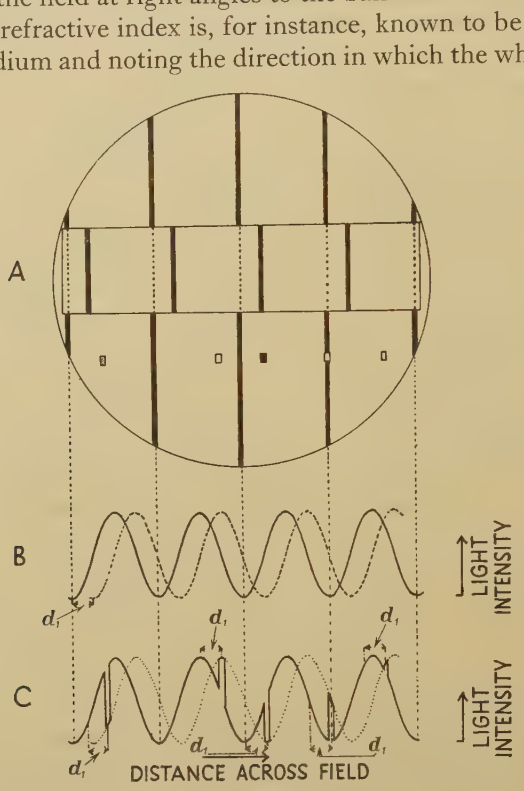


FIG. 4. Simple objects of known geometrical form in the field of view crossed by interference bands. A, in the upper section interference bands are seen displaced in the large strip object of constant optical thickness; in the lower section five small objects of the same optical thickness are shown situated on a line at right angles to the bands, and only part of a band will be seen in them. B, light intensity distribution across clear background field (full line) with band separation d; the light intensity across the object (broken line) is similar in shape but displaced through a distance d_1. C, light intensity distribution (full line) across a line containing the five similar small objects is simply constructed from the light intensity in the clear background field (dotted line); all distances d_1 are equal.

has been displaced. The central white band then viewed through this object is moved in the direction of decreasing optical path difference in the microscope field.

(*b*) *Small uniform object.* When the object is of uniform thickness and small in size compared with the distance between bands, the band system is similarly shifted, but only a small part of a band is visible in the area of the object. Small objects located at various positions in the field (fig. 4, A, lower part) will appear with approximately uniform light intensities varying in magnitude with their position in the field, as shown in fig. 4, C. Thus, as a small object

moved across the field its intensity will alternately be greater and less than the background, and at two positions between adjacent dark bands it will be approximately the same as the background, and consequently the object will vanish from view. The optical retardation of the small object may be measured as above. Thus, it is moved in the appropriate direction at right angles to the band system until the white band of zero path difference appears in it; the distance of this band from the white band in the background can then be determined.

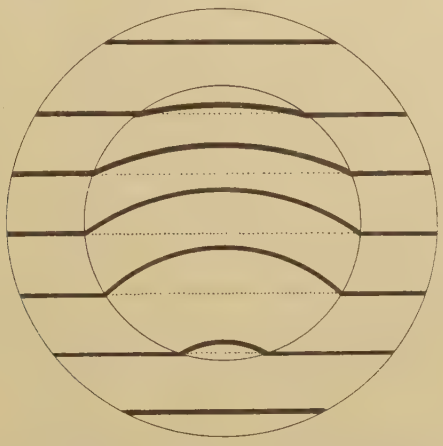


FIG. 5. This shows the interference bands in a lenticular object of uniform refractive index.

(*c*) *Large non-uniform objects.* When a large object, in which the optical path length is varying, is placed in the field, the interference bands are displaced and are no longer straight. For example, the bands produced by a lenticular object of uniform refractive index lying in the plane of the field are shown in fig. 5. At any chosen point on a band in the image of the object the displacement from the corresponding clear-field band (interpolated), in a direction at right angles to the clear-field bands, is a measure of the o.p.d. at that point. Hence, following a given dark band across the object, the displacement of this band measures the variation in o.p.d. along the band. When the object is of uniform thickness, but contains a varying concentration of substance, the gradient at a point on the band is proportional to the concentration gradient of substance at that point in a direction parallel to the clear-field bands. When the variation in o.p.d. is not too rapid, the shape of any band corresponds to a graph of o.p.d. along a line drawn across the object parallel to the clear-field bands.

b. Background field uniformly illuminated

When there is no variation in optical path difference over the field, the field appears uniformly illuminated. Under these conditions the bands seen in objects several wavelengths thick are contour bands of optical thickness μt;

that is, along any band the optical thickness of the object is constant in value. The bands appearing in a homogeneous spherical object of optical thickness 3λ (fig. 6, A), a wedge, increasing from zero to 3λ (fig. 6, B), and an irregular object (fig. 6, C), are shown in a uniform field at maximum brightness. These objects are assumed to be large compared with the wavelength of the light used and illuminated with parallel light. It is also assumed that the differences

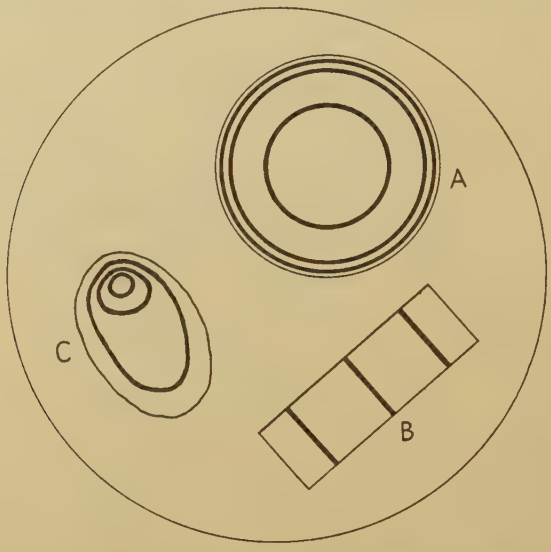


FIG. 6. The appearance of objects in a uniform field of view, with band spacing infinite, and at maximum brightness. A, a homogeneous sphere of o.p.d. 3 wavelengths (λ). B, a wedge increasing in o.p.d. from 0 to 3λ. C, an irregular object of maximum o.p.d. 3λ. The dark bands shown are contour lines of equal o.p.d.

in refractive index between the object and the medium is small, so that the distribution of light intensity in the image is not altered appreciably as a result of light refraction. The bands in the wedge-shaped object lie parallel to its axis. It will be noted that the bands in the image of the sphere are more closely spaced towards the edges; this follows from the increased rate of variation of optical path with distance from the centre of the sphere. Hence, in objects of uniform geometrical thickness, for example, a thin biological section, the way in which the band separation varies across the object, gives an indication of the nature of the concentration gradients of substances other than water in direction at right angles to the bands in the object.

3. *Measurement of fractional part of optical path difference*

When the field is crossed by interference bands with monochromatic light a simple way of measuring the fractional part of the optical path difference is to move the object across the field, or move the bands across the field by means of the 'phase screw' until any desired portion of the object is at a minimum of light intensity; the distance from this portion of the object to the correspond

ing background dark band must then be measured (e.g. by micrometer eyepiece, &c.). This method works quite well if the portion of the object to be measured is relatively large and of even optical thickness so that an appreciable part of a dark band can be seen in it. The accuracy attainable is then that of setting the centre of the dark band on a cross-hair and this accuracy will depend on the contrast. The accuracy is usually quoted as one-tenth of a band and, with care, one-fiftieth (e.g. Jenkins and White, 1951, p. 246).

It is found convenient in practice when making the measurements of optical thickness to use one of the two dark bands seen in white light adjacent to the white central band, since they can be set more accurately on any desired portion of the object. The illumination is then changed to monochromatic light when estimating more precisely the fractional part.

When the object is small compared with the band separation, only a small portion of a dark band can be seen in it. Similar considerations may apply to portions of a large heterogeneous object. The accuracy attainable in setting an object at a minimum of light intensity is then reduced. The judgement of the position of minimum intensity in the object is also made more difficult by the variation in light intensity of the background to the object. Thus the object may look apparently darker due merely to the increased brightness of the surrounding field. The accuracy in setting the object to a minimum light intensity could be improved by using an objective method, e.g. a photoelectric detector.

For small plate-like objects of constant optical thickness a method suggested to us by F. H. Smith can be used. It has been shown above that as a small object is moved across the field there are two positions between adjacent dark bands where the object can be set so that it disappears. The setting from which the fractional part can be determined is very critical since the brightness of the object and field vary in opposite directions. This method is not often used since biological objects rarely conform to the requirements of even optical thickness. The above method is comparable to one proposed by Dyson (1950), which gives an accuracy of about one-fortieth λ in the optical thickness. The latter method is not limited to objects of even optical thickness, but requires the manufacture of interference plates having 'scratches' of even optical thickness along their length and these may be difficult to obtain.

In interference microscopes employing doubly refracting components it is often possible to make accurate and direct measurements of o.p.d. at individual points in the cell by employing additional optical elements. For example, Smith has described (private communication) the use of a Nakimura plate for this purpose, and Dyson (1953) has modified the interference microscope of Lebedeff (1930) for point measurement in cells and under good conditions claimed an accuracy of $\pm 0{\cdot}003\lambda$.

The uniform objects shown in fig. 4 shift the interference bands in a direction at right angles to the bands without changing the shape of the bands. Thus the o.p.d. of the small object (see fig. 4, c) may be determined by measuring any of the equal distances marked d_1. The o.p.d. at a chosen point

in an object of varying o.p.d. can be determined by measuring the distance, in a direction at right angles to the bands, between the chosen point on the band in the object and the point on the corresponding background band where the light intensity is the same: e.g. see Fig 7.

Comparison of Phase Contrast and Interference Contrast Microscopy

The Dyson interference microscope has several advantages (Dyson, 1950) over the ordinary phase contrast microscope. (i) The object can be viewed with variable contrast by operation of the phase screw, and the path difference between the two interfering beams thus adjusted in an instant to give maximum visibility to the particular cell structure being observed. (ii) As described later on, measurements of optical thickness can readily be made. (iii) In the phase contrast microscope the variation of light intensity in different parts of the image is caused by a redistribution of light energy in the image field. For example, an optically retarding region, which appears dark in positive phase contrast, is surrounded by a bright 'halo'. This optical artifact sometimes makes precise interpretation of the image difficult and is entirely absent in the interference microscope. Furthermore, the phase contrast image does not give a true picture of the variation in optical path length over an object which is large compared with the resolving power. For example, a uniform plate will appear dark only near its edge and a uniform spherical cell may appear to be of less optical thickness at its centre than nearer the edge. In so far as the object lies approximately in one plane not large compared with the depth of focus of the microscope, the image in the interference microscope gives an easily interpreted picture of the variation of optical path length in the object. The only ambiguity arises from parts of the object being out of focus or insufficiently resolved. Such ambiguity occurs in all forms of light microscopy. Thus, in the interference microscope when the band spacing is infinite, a uniform plate will appear uniformly dark (or light) with respect to the background; when the band spacing is finite the variation in intensity in the image of the plate can be simply interpreted. The redistribution of light energy arises in different ways in interference and phase contrast microscopy. In the former system any light energy removed from the image is to be found in a reflected interference band system which, since it is not viewed, does not give rise to any confusion. (iv) It has been found in practice with relatively thick and heterogeneous plant cells, that the morphological features can be much more easily distinguished in the living cell by means of interference than by phase contrast microscopy. This is partly due to the absence of confusing halos in the interference microscope.

Experimental Methods and Materials

The measurements were made with a microscope designed and constructed by Mr. J. Dyson, with objective N.A. 0·75 and condenser N.A. about 0·5. Köhler illumination was used with white tungsten light or monochromatic

46mμ light from a B.T.H. 250 watt Hg compact-source arc with Wratten 7A and 58 filters.

Cells were photographed at magnification ×68 on to either Ilford Pan F 5 mm. film (exposure time about 1 second) or Kodak Microfile Pan 35 mm. lm (exposure time about 5 seconds), by using an R. J. Beck observing and ocusing adaptor. Observations on *Amoeba* were made with an interference nicroscope, N.A. 0·2, constructed by W. E. Seeds according to the prototype nodel designed and built by Huxley (1952). The two interfering beams, the eam through the object and the comparison beam, are produced by doubly efracting optical components (Wollaston prisms) (Smith, 1950). Film den-ities were recorded by a microdensitometer (Walker, 1953); the refractive ndices of the media used for immersing cells were measured on an Adam Hilger Abbe-type refractometer.

Measurements of optical path differences

In the measurements on the heterogeneous pollen grains, where the o.p.d.s ange from about 0·3 to about 3λ, the following procedure was adopted. The .p.d.s in the various regions of the image of the cell in a field containing inter-erence bands were first mapped out roughly on a sketch of the cell by using hite light. Then, from a photomicrograph taken with monochromatic light λ = 546 mμ), with an evenly illuminated field, an enlargement on to bromide aper was prepared. From the enlargement a narrow strip was cut out with ength at right angles to the band system from a region of the clear field ad-acent to the cell. This strip was then divided up into intervals equal to one-enth of the band separation. By sliding the strip alongside any chosen part of he image of the object, the light intensity in the image and strip can be natched by eye. The fractional part of the o.p.d. may thus be measured with n accuracy of about one-twentieth wavelength.

A more accurate method, employing a microdensitometer, was used for easuring the o.p.d.s in tissue culture cells and sperm heads, in which the .p.d.s were about 0·1 wavelength. The band separation was adjusted until the ell, e.g. the living chick fibroblast in fig. 7, A, or ram sperm, fig. 7, C, occupied position about midway between a light and dark band; i.e. on a part of the and system where the rate of change of light intensity with distance across he field is at a maximum. From a photomicrograph the film density was ecorded (fig. 7 B) along any chosen line in the image of the cell by means of recording densitometer and a suitable 'blank' trace taken to fill in the portion f the field occupied by the cell; such a blank trace is not required for the elatively small ram sperm heads which obscure less of the background field fig. 7, D). The o.p.d. at a chosen point in the cell may then be determined by easuring the distance D between the chosen point and the point on the cor-esponding band where the light intensities are equal. The o.p.d. in wave-engths is then given by the ratio D/d, where d is the measured band separa-ion. This method has the following advantages. (i) The accuracy attainable s about ±0·005 wavelength. (ii) From a single photograph, taking about

1 second, the o.p.d.s at all points in the cell can be calculated. This is an adv vantage in measurements on living and moving objects. (iii) The o.p.d. at an point along the chosen line in the cell may be determined by measuring th appropriate distance *D*. Hence the separation between the trace through th cell and the superimposed trace viewed in a direction at right angles to th bands provides an immediate representation of the variation in o.p.d. an therefore dry mass per unit area along the chosen line through the cell. Whe the object is of constant geometrical thickness, concentration gradients alon any chosen line may be conveniently determined.

This method requires the illumination across the field (in the absence c interference) to be even. This can be simply checked and, if necessary, a blan trace obtained from the appropriate region on a second photomicrograp taken with the same exposure. A second photomicrograph is essential if th structure of interest lies in the centre of a large object occupying most of th field. These measurements may be made equally well with a microphotomete which records light transmission through the film rather than the density c the film, and may also be made photoelectrically by scanning the image plan with an aperture of the appropriate size.

2. *Measurements of cell total dry mass*

The o.p.d. which is proportional to the dry mass per unit area often varie over the cell and the total dry mass of the cell may be determined by integratin the o.p.d. over the area of the projected cell image. The dry mass of the polle grain was determined by first dividing up the projected cell image viewed a suitable enlargement into areas over which the o.p.d. was approximately con stant; the dark bands seen inside some pollen grains often formed convenien areas. The products of o.p.d.s and chosen areas were then summed over th total projected area to give approximately the total dry mass of the cell.

For the measurement of total masses of sperm heads and fibroblast cells i tissue culture the appropriate average o.p.d. was determined from a series o traces across the cell by using the densitometer trace displacement metho (fig. 7). The product of average o.p.d. and cell projected area then gave th total dry mass.

Fig. 7 (plate). The photomicrographs in this figure, and in figs. 9 and 11, were taken wi the Dyson interference microscope, objective of N.A. 0·7, condenser of N.A. about 0·5. Th primary magnification was ×68. 35 mm. film was used.

A, living interphase chick heart fibroblast mounted in Tyrode solution. Ilford Pan F film

B, film density (ordinate) from negative recorded along the line indicated in A. The dott curve is filled in from a suitable 'blank' field, nuclear dimensions α, cytoplasmic dimensions The o.p.d. at any chosen point on the line is determined from the distance *D* measured right angles to the bands.

C, ram sperm, fixed in 10 per cent. neutral formalin, mounted in water. Kodak Microf Pan film.

D, film density (ordinate) from negative recorded along the line indicated in C; cell dime sions α. The o.p.d. at any chosen point on the line is determined from the distance *D*.

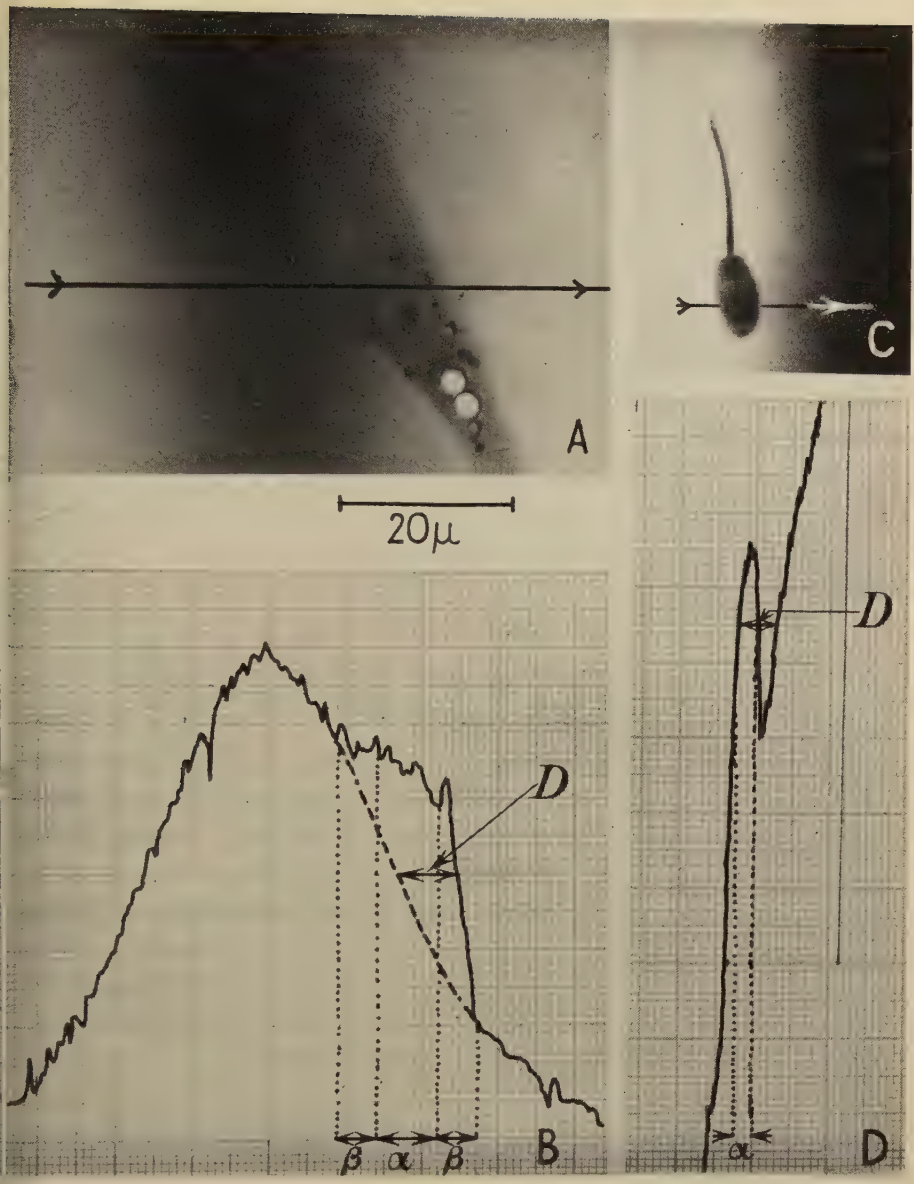


FIG. 7

H. G. DAVIES, M. H. F. WILKINS, J. CHAYEN, L. F. LA COUR

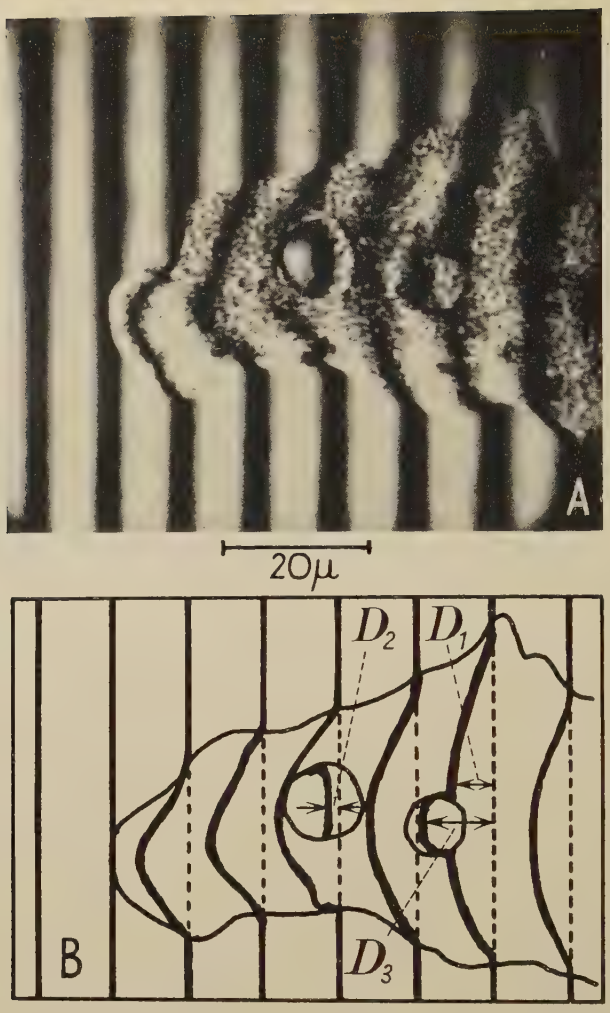


Fig. 8

H. G. DAVIES, M. H. F. WILKINS, J. CHAYEN, L. F. LA COUR

. *Materials*

(*a*) Buds from *Tradescantia bracteata* at various stages of development were issected; one-half of an anther was placed in aceto-carmine and examined for asy identification of the stage, and a preparation in 5 per cent. sucrose was nade of the other half for measurement with the interference microscope.)ne anther, just before anthesis, was fixed in acetic alcohol, taken down to vater, and washed, and the pollen grains digested under the microscope for hour at room temperature with a 0·2 per cent. solution of ribonuclease supplied by Armours) in M/40 $NaHCO_3$. Further digestion for 2 hours was nade with a 0·5 per cent. solution of trypsin (supplied by B.D.H.) in Tyrode olution. Observations on staminal hairs from *T. bracteata* mounted in 5 per ent. sucrose were also made.

(*b*) Fragments of heart from 11 to 12-day chick embryos were grown at 7° C. by a modification of the hanging drop method devised by H. B. Fell for ytological studies. After 24–48 hours the coverslip bearing the explant surounded by a single layer of cells was washed thoroughly with warm Tyrode olution for 1 minute to remove the liquid protein medium, and mounted for neasurement at room temperature in Tyrode on a glass slide with 0·5 mm. pacers between slide and coverslip.

(*c*) Living ram sperm (kindly supplied by Dr. A. Walton) were measured vhen immersed in Tyrode solution; measurements were also made on ram perm fixed in 10 per cent. neutral formalin Ringer (solution prepared by iluting commercial formalin 1:9 with Ringer; the commercial material was eutralized by shaking with powdered calcium carbonate). *Amoeba* were indly supplied by D. M. J. Ord and measurements were made in Chalkley's olution.

Results and Discussion

Section I

. *Amoeba*

Living *Amoebae* were photographed, with Dr. W. E. Seeds, on the Huxley nterference microscope in a field containing a number of interference bands see fig. 8, A). The bands in the clear field are moved to the left (direction f decreasing optical path difference in the microscope field; see section 1 (*a*) bove) within the area of the *Amoeba*. The *Amoeba* viewed in this way corresponds with the lenticular object discussed in the previous section 1 (*c*). Along any dark band the overall o.p.d. between the two interfering beams

---

Fig. 8 (plate). A, living *Amoeba* in Chalkley's solution. Ilford Pan F film. The photomicrograph was taken with the Huxley interference microscope, with objective of N.A. 0·2. The rimary magnification was ×24.

B, schematic diagram of interference bands seen in background field and image of *Amoeba*. The o.p.d. varies from zero at the cell edge to a maximum near the cell centre, e.g. the o.p.d. $_1/_d$ is about 0·6, where d is the band separation. In the contractile vacuole the o.p.d. is bout 0·1 λ ($_{d_2}/_d$), while in the food vacuole it is about 1·0 λ ($_{d_3}/_d$).

is constant, and at any point the displacement of the band to the left is a measure of the dry mass.

The o.p.d. is proportional to the mass per unit area and is seen to vary from zero at the edge of the cell to a maximum value (e.g. about $0{\cdot}6\lambda$ corresponding to D_1 in fig. 8, B) near the centre of the *Amoeba*. The shape of the band is approximately a graph of dry mass per unit area along a line across the *Amoeba* (see section 1 (*c*)).

In the contractile vacuole the o.p.d. is about $0{\cdot}1\lambda$ (D_2), while in the food vacuole it is about $1{\cdot}0\lambda$ (D_3). Values for the concentrations of substance could only be determined from measurements of geometrical thickness. However, it is obvious that the concentration of material other than water is much less in the contractile vacuole and greater in the food vacuole than in the rest of the protoplasm. By an integration procedure the total mass of an *Amoeba* may be determined approximately and such data may prove useful in following the results of enucleation experiments. The approximate dry mass of the nucleus may be readily obtained. Extreme granularity in some *Amoebae* may introduce errors.

2. *Tradescantia bracteata*

(*a*) *Pollen grain development.* The meiotic divisions of the pollen mother cells result in the formation of four daughter cells (the tetrad) which separate to form individual microspores containing a single nucleus and numerous granules (fig. 9, A). These cytoplasmic granules are about $1{\cdot}0\mu$ in diameter and are thought to be of a protein nature (La Cour, 1949). They occur at meiosis and persist in the pollen grain till the formation of the vacuole (Sax and Edmonds, 1933). The concentration of substance other than water in the granules was roughly determined as follows. In general, if ϕ_1 is the optical retardation of a body in the cell seen in projection, that is including the material above and

---

Fig. 9 (plate). Pollen grains of *Tradescantia bracteata*, isolated in 5 per cent. sucrose, at various stages of development. Ilford Pan F film.

A, individual microspore with central nucleus and granules in cytoplasm (stage 1).

B, shows pollen grain with a single spherical nucleus (dark) and vacuole (light) of similar dimensions, situated at the ends of the cell (stage 2).

C, two pollen grains now larger than in *B*, each with a single vacuole. A nucleus is located at the end of each cell. Pores are seen in the cell membrane (stage 3).

D, pollen grain with two large vacuoles (one dark, one light), after nuclear division. The generative nucleus clearly shows a single nucleolus with central core and surrounding 'middle zone'; the vegetative nucleus appears to contain two small nucleoli, each with a 'middle zone' (stage 4).

E, the generative nucleus and relatively larger vegetative nucleus with nucleolus are centrally placed; the vacuoles, diminished in size, are at the ends of the pollen grain (stage 5).

F, pollen grain with spherical vegetative nucleus and differentiating generative nucleus (classed as stage 7).

G, pollen grain near maturity, in which the generative nucleus has assumed its characteristic sickle shape (stage 7).

H, I, staminal hair cells from *Tradescantia bracteata* isolated in 5 per cent. sucrose. Ilford Pan F film. In each cell there is a small dark nucleolus with surrounding lighter 'middle zone' (located by arrows).

The scale under G refers also to figs. A to F.

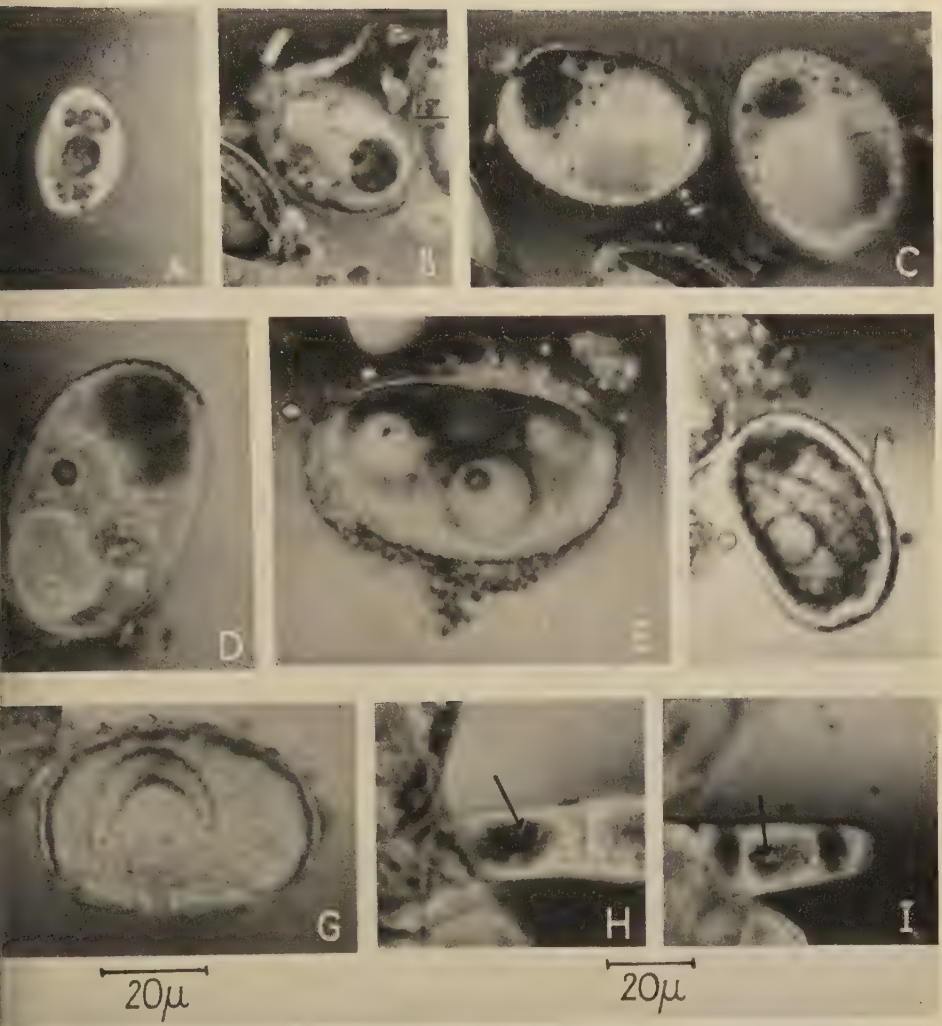


FIG. 9

H. G. DAVIES, M. H. F. WILKINS, J. CHAYEN, L. F. LA COUR

below it, and ϕ_2 the optical retardation in an adjacent clear region of the cell, then the concentration of the body in excess of that of the cytoplasm is given by $(\phi_1-\phi_2)/\chi t$, where χ has been previously defined (p. 274) and t is the thickness of the body. This assumes that the thickness of the cell does not vary appreciably in the region of the body and this will be true for the granules in the microspores. The above concentration of the granules was found to range from 70 to 100 gm./100 c.c. (using $\chi = 0{\cdot}18$). The average concentration of the microspore is shown later (fig. 10, B) to be about 20 gm./100 c.c. and this must be added to the above values to obtain the absolute concentration of the granules. Hence, as dry protein has a maximum concentration $\sim$ 127gm./100 c.c. (see p. 275), it is concluded from these rough measurements that the cytoplasmic granules are highly concentrated.

With the formation of the vacuole which is a region of relatively low optical retardation at one end of the cell (fig. 9, B), the nucleus, seen as a dark spherical body, migrates to the other end of the cell. Later in this stage the volumes of the cell and vacuole increase and pores in the cell wall can be seen (fig. 9, C). Before nuclear division, the nucleus occupies a central position with a vacuole on each side. The optical retardations of the two vacuoles seen as dark and light areas in the cell after nuclear division (fig. 9, D) were approximately equal, the contrast depending on their position in the band system.

Since the interference microscope had the advantages over the phase microscope of (i) absence of the 'halo effect', and (ii) variable contrast, it was relatively simpler to interpret the nucleolar structure seen, for example, in the generative nucleus (fig. 9, D). The nucleolus was shown to consist of an outer region of higher concentration (that is, dry mass per unit volume) than any other part of the interphase nucleus; it contains a central region of relatively low concentration. The nucleolus is surrounded by a circum-nucleolar zone which has a lower concentration than either the outer nucleolar region or the remainder of the nucleus. Similar nucleolar structures, together with circum-nucleolar zones earlier referred to as 'middle zones', have been previously deduced from observations with phase-contrast and ultraviolet microscopy together with staining techniques in the fixed meristem cells of *Vicia faba* and in living *Tradescantia* staminal hair cells (Chayen, Davies, and Miles, 1953). The middle zone in living *Tradescantia* staminal hairs was seen, with the interference microscope, to be a region of relatively low optical retardation (figs. 9, H and I).

At a later stage during pollen grain development (fig. 9, E) the two nuclei can be seen in a central position in the cell with the generative nucleus nearest the cell wall and the relatively larger vegetative nucleus containing a nucleolus. The vacuoles are now diminishing in size and will eventually disappear. The generative nucleus changes its form (fig. 9, F) and assumes a characteristic sickle shape (fig. 9, G). In the latter two cells the optical retardation amounts to several wavelengths, and several interference bands can be seen inside the cells.

Measurements of the dry mass of individual living cells were made at stages

during development from the microspores to the mature pollen grains. T time duration of the various stages depends partly on environmental conc tions. For purposes of graphical presentation (fig. 10, A and B) cells whose d masses had been determined were placed into well-recognizable stages and t time intervals between them taken arbitrarily as equal. The upper arro

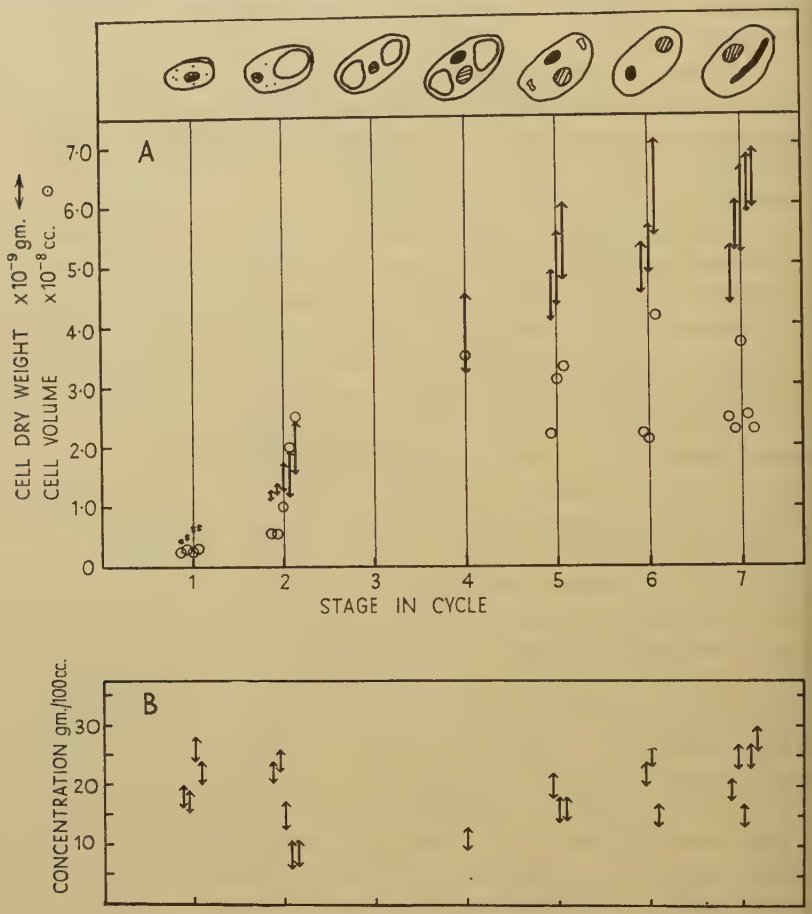


FIG. 10. This shows *A*, the increase in cell dry mass (arrows), total cell volume (circles), and the increase in average cell concentration, at various stages in pollen grain developmen *Tradescantia bracteata*. The upper arrows indicate the true mass *A* or concentration *B* if sucrose enters the cell, and the lower arrows indicate the true mass *A* or concentration *B* if fluid medium of the cell has an additional 5 per cent. sucrose.

(fig. 10, A) indicate the true dry masses of the pollen grains calculated fr equation (9), which allows for immersion in 5 per cent. sucrose and assur that no sucrose enters the cells. In order to show the magnitude of the corr tion factor the value for the dry mass was also calculated (lower arro neglecting the correction term $(\chi'/\chi m')$. The arrowed limits also serve anot useful purpose. Conceivably sucrose is entering the cell and it is possible t

he concentration of sucrose in the fluid medium in the cell might become qual to that of the external medium. It would then be incorrect to add the orrection term, and the true dry mass (before immersion) would be indicated y the lower arrow. The cell volumes (circles) were calculated on the assumption that the cells were ellipsoidal. Owing to the peculiar shape of the microspores the calculated volumes are only rough approximations.

The measurements of total dry mass in the pollen cell cannot be made ccurately on account of the granularity and irregularity of the cells, but as arge changes of mass are observed rough measurements are adequate.

The dry mass increases about ten times during development from the microspore to the mature pollen grain, the greatest percentage increase in dry weight occurring during the formation of the vacuoles. From stage 4, when the nucleus has divided, and onwards, the dry weight is still increasing, but less rapidly. The cell volume then appears, on average, not to increase, indicating an increase in concentration of material in the cell. The average concentrations of dry material in the cells, calculated from the data in fig. 10, A, are shown in fig. 10, B.

An analysis of the cells at stage 2 shows that the cell volume is increasing at a greater rate than cell dry weight, so that there is a marked drop in average concentration of dry substance in the cell. The larger cells with the lower average concentration contain large vacuoles, so that it is likely that the concentration of the protoplasm itself does not diminish when the cell volume increases. It might be suggested that the formation of the vacuole in the expanding cell prevents the concentration of the protoplasm falling below the value at which it can function. In this connexion it may be useful to recall the behaviour of the contractile vacuole in some fresh-water protozoa, where it is believed that a function of the vacuole is to excrete water, thereby preventing the protoplasm from becoming waterlogged.

(*b*) *Enzymatic digestion of mature pollen grains.* According to Painter (1943), whose conclusions are based on visual estimations of the staining intensity before and after digestion with ribonuclease, the mature pollen grains store 'large amounts' of ribonucleic acid. Cell interferometry may be used to measure the amounts of those substances which can be removed differentially from cells. The dry masses of four mature pollen grains were determined before and after successive digestions with ribonuclease and trypsin (table 2). It appears that between 4 and 14 per cent. ($200–700 \cdot 10^{-12}$ gm.) of the contents of the mature grain are digested away by ribonuclease, while about 40 per cent. of the initial cell contents remain after the trypsin digestion. The mass of the remaining material only partly consists of cell wall since the outline of the generative nucleus and, more rarely, the vegetative nucleus could still sometimes be distinguished. Using bulk biochemical methods, Ogur, Erikson, Rosen, Sax, and Holden (1951) obtained a value of 1700×10^{-12} gm. for the ribonucleic acid content of the mature pollen grains of *Lilium longiflorum*. The volume of these cells, obtained by rough comparison, is seen to be many (about 10–30) times greater than that of the corresponding cells in *Trades-*

cantia bracteata. It can be shown that the mass of material per mature pollen grain in *Tradescantia* removed by ribonuclease would give an optical extinction of about 3·0 at 265 mμ if it absorbed like ribonucleic acid, when light rays travel through the whole thickness of the cell. Photomicrographs of these cells at the wavelength 265 mμ were seen to be densely absorbing (see also Uber 1939). The experiments described here were of an exploratory nature and meant to show the general feasibility of the methods. No investigation was made of the errors which may arise from a variety of causes, for example, in complete removal of ribonucleic acid and removal of substances other than ribonucleic acid. Hence the results obtained must be viewed with caution.

TABLE 2

Dry mass changes during enzyme digestion of mature pollen grains

Cell no.	*Dry mass* 10^{-12} *gm.*	*Dry mass after ribonuclease* 10^{-12} *gm.*	*Dry mass after trypsin*	*Per cent. loss after ribonuclease*	*Per cent. loss after trypsin*
1	5070	4870	2270	4	55
2	4890	4210	1970	14	60
3	5140	4910	2150	4·5	58
4	5120	4500	1990	12	61

(*c*) *Mitosis in staminal hairs*. A series of photomicrographs (fig. 11) were taken at various stages during mitosis in a staminal hair cell from *T. bracteata*. Interphase nuclei in the staminal hairs have a finely granular appearance, pro phases (fig. 11, A and B) a coarser granularity which, as mitosis develops changes into thread-like chromosomes (seen at late prophase in fig. 11, and D). After the two groups of chromosomes have separated (fig. 11, F onwards), large concentration gradients were observed in the spindle region between the two chromosome groups. These gradients were associated with a markedly high concentration of substance in the centre of the spindle and a few minutes later the beginnings of the cell wall appeared as a fine line of specially high concentration in the centre of this region (fig. 11, H). The dry mass per unit area along the long axis of the cell (fig. 11, H) is roughly shown in fig. 12; since the thickness of the cell is approximately constant, fig. 12 also indicates the variation in average concentration along the line in the cell. In figs. 11, I and 11, J, taken at different positions in the band system, the new cell wall appears with different contrasts, as a light, or as a dark, line.

At the stage when the cell wall was being formed, spindle fibres might just be

Fig. 11 (plate). Stages in the mitotic division of staminal hair cell of *Tradescantia bracteata*. Ilford Pan F film.

A, B, cell at early prophase.

C, D, E, cell at late prophase.

F–K, cell at telophase.

L, early reconstruction.

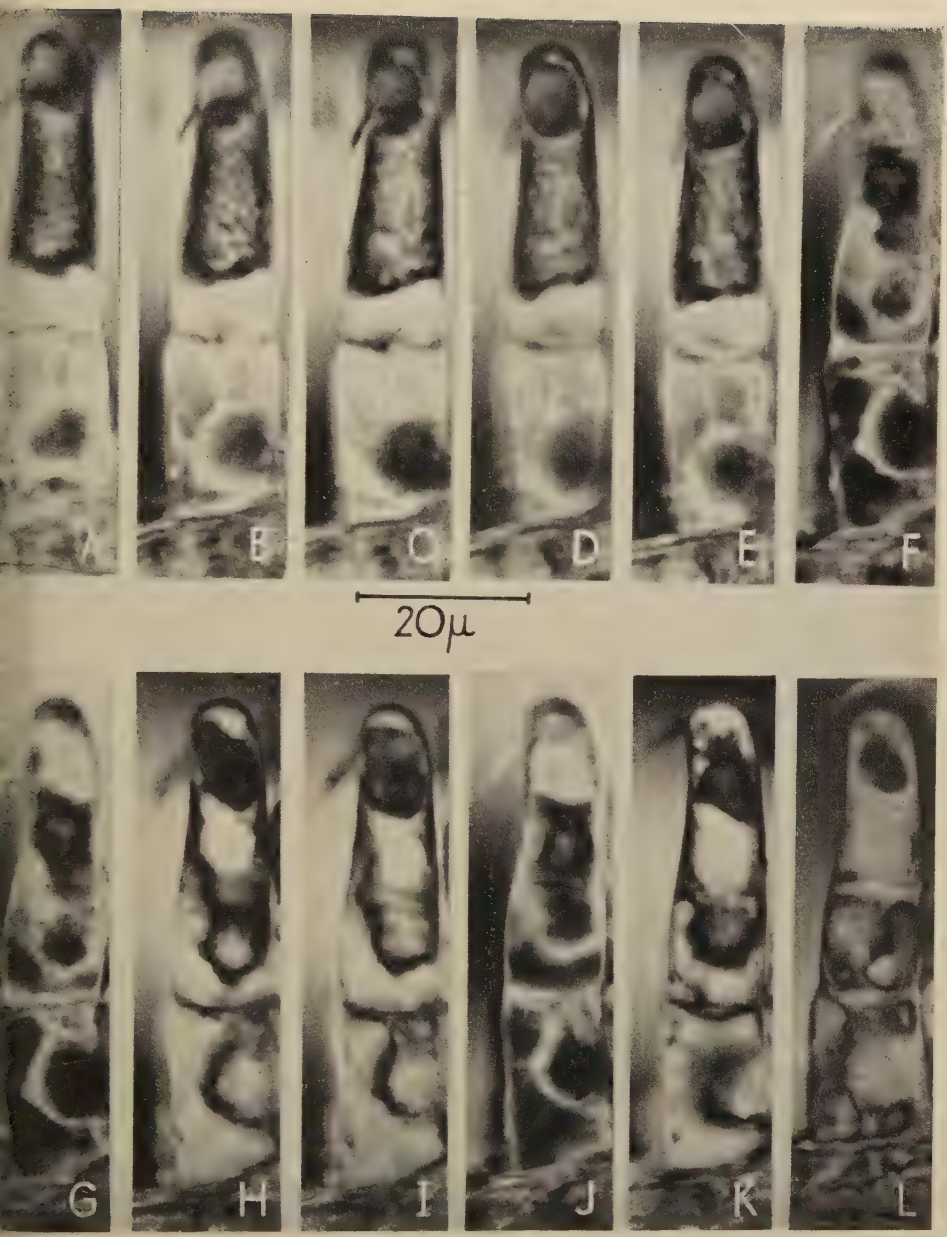


FIG. 11

H. G. DAVIES, M. H. F. WILKINS, J. CHAYEN, L. F. LA COUR

seen lying roughly perpendicular to it. The cell wall then grew sideways towards the walls of the staminal hair (fig. 11, K and L) until it completely separated the two daughter cells. It might be suggested that these concentration gradients indicate the movement of material towards the centre of the spindle region and, when sufficient concentration is attained, deposition of the cell wall begins.

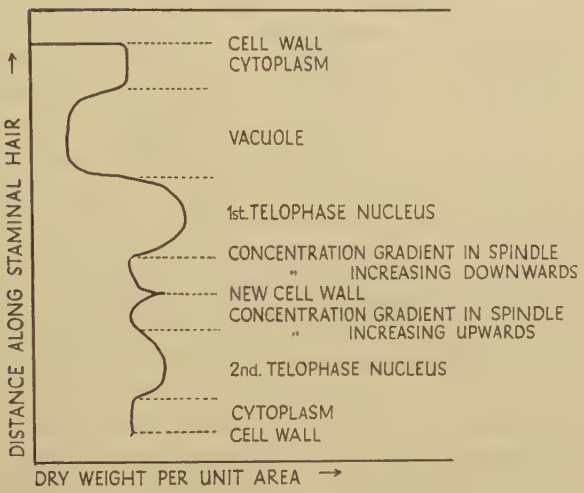


FIG. 12. The dry mass/unit area along the long axis of the hair cell in fig. 11, H is shown roughly. The mass/unit area is proportional to the concentration of dry material since the thickness is approximately constant.

3. *Chick fibroblasts*

In the projected nuclear area of living interphase chick heart fibroblasts grown in tissue culture, the content of material absorbing at a wavelength 265 mμ has been shown to vary from 4 to 12×10^{-12} gm. This variation is associated with the synthesis of DNA during interphase (Walker and Yates, 1952). It should be possible, by cell interferometry, to measure approximately the nuclear protein content by taking the difference between the total dry weight and ultra-violet absorbing content. This would assume the nucleus to consist largely of desoxyribose nucleic acid and substances with similar absorption, together with protein. As a result of such measurements during stages in interphase the changes in nuclear protein content may be correlated with the amounts of material absorbing at 265 mμ.

Preliminary results on the dry masses of three living cell nuclei in Tyrode solution obtained by using the trace displacement method (fig. 7, A and B) are summarized in table 3. The average o.p.d. is about 0·1 (col. 3) and the dry masses of the contents of the projected nuclear area vary from 28 to 36×10^{-12} gm. (col. 4). Normally a small additional correction (col. 5) is required to allow for immersion of the cells in Tyrode solution (χ' is about 0·16), and this has been calculated (equation (9)), assuming the cell thickness to be

2 to 4μ. However, when the nuclear protein content is being estimated it may be more accurate to neglect this correction term since it may represent the weight of low molecular weight material (salts, &c.) already present in the nucleus.

TABLE 3

Dry mass of nuclear area of chick fibroblasts

Cell no.	*Nuclear projected area* μ^2	*Average optical thickness wavelengths*	*Dry mass* $\times 10^{-12}$ *gm.*	*Additional correction-factor* $\times 10^{-12}$ *gm.*
1	80	0·13	32	1·5–3
2	78	0·12	28	1·5–3
3	128	0·09	36	2·5–5

4. *Ram sperm*

Interferometric measurements on the heads of living ram sperm (table 4) showed on the average an optical retardation of about 0·07λ and an average dry weight of 6·9 10^{-12} gm. (using $\chi = 0{\cdot}18$). The additional correction factor resulting from the Tyrode immersion, assuming a thickness of 1·0μ, is $0{\cdot}3 \times 10^{-12}$ gm. The average value for the DNA content of the haploid ram sperm heads determined from the ultra-violet absorption at 265 mμ is $2{\cdot}9 \times 10^{-12}$ gm. (Walker and Yates, 1952); this is in good agreement with the bulk biochemical values of $5{\cdot}4$, $6{\cdot}1 \times 10^{-12}$ gm. for the diploid liver cells of the same species (Vendrely and Vendrely, 1949). The combination of these data gives a value of 40 per cent. for the ratio of DNA to total dry weight and is in good agreement with the ratio of 45 per cent. obtained from bulk biochemical methods by Mann (1949), who gives 4 per cent. as the fraction of nucleic acid phosphorus in ram sperm head.

TABLE 4

Dry mass of ram sperm heads

Cell no.	*Projected area in* μ^2	*Average optical thickness in wavelengths*	*Dry mass* $\times 10^{-12}$ *gm.*
1	30	0·07	6·3
2	33	$0{\cdot}07_5$	7·4
3	34	$0{\cdot}06_5$	6·6
4	30	$0{\cdot}07_5$	6·8
5	32	$0{\cdot}07_5$	7·3
			Av. 6·9

SECTION II: VARIATION OF REFRACTIVE INDEX OF IMMERSION MEDIUM

1. *Matching the refractive index when using the phase microscope*

The refractive index of microscopic particles can be measured by immersing them in a series of liquids of known refractive indices, the particle disappearing from the field of view when the refractive index of the medium is equal to

iat of the particle. Oettlé (1950) used the phase contrast microscope, which he ound was much more sensitive for determining small o.p.d.s than the con-entional Becke line method used by mineralogists. He measured the refrac-ve indices of dried blood-cells, bacteria, sperm, &c., using a series of oily efractive index mixtures. Barer, Ross, and Tkaczyk (1953) have recently rawn attention to the fact that from such measurements of cell refractive idex made with the phase microscope, the concentration of dry material in ie cell may be directly determined (compare equation (3)); no measurement f cell thickness (or projected area) is required. An important aspect of this ietliod is the use of suitable solutions of proteins in which living cells may be mmersed and remain apparently undamaged.

This technique works well when determining the refractive index of those arts of the cell which are largely in contact with the immersion medium, e. the peripheral regions of cells or different parts of a linear cell of small iameter (e.g. a sperm) or any object that has uniform refractive index. Thus, mall cells of uniform refractive index, but with irregular shape and indeter-iinate dimensions, are readily measured. The inner regions of non-uniform ells cannot be measured quantitatively by this method. Hence chromo-omes, nucleoli, &c., cannot readily be studied inside rounded cells.

. *Varying the refractive index when using the interference microscope*

(*a*) *Résumé.* We will summarize the data which can be obtained by inter-erence microscopy when the cells are observed in a medium in which they ither grow well or are known to survive. It has been shown (compare equa-on (1)) that the total dry masses of cells in water may be determined from ie measured projected area and o.p.d. without a knowledge of cell thickness. Vhen the cells are immersed in an isotonic medium, which differs in refractive idex from water, an additional correction term involving the thickness of the ell appears in the relationship connecting dry mass with o.p.d. (compare quation (7)). However, this term is often small and it is then sufficient to iake only a very rough estimate of cell thickness. The concentration of dry iaterial, as distinct from total amount, in a cell or part of a cell may be deter-iined from the dry mass measurement if the thickness, and hence the volume, an be measured. Thus the concentration of bodies inside cells can be deter-iined if the linear dimensions of the body and the cell can be measured (cf. 289). However, it is sometimes not possible to measure accurately the thick-ess of cells, for example fibroblasts in tissue culture, sperm heads, &c., the iickness of which is not many times greater than the depth of focus of the iicroscope. Hence this technique, although it can be used for total dry masses, annot be used for concentrations in such cases.

(*b*) *Combination of o.p.d. measurement with variation in refractive index of the iedium.* By combining the total dry mass measurement from interference iicroscopy, and the concentration measurement from phase microscopy, the iickness of the cell may clearly be determined (Barer and others, 1953).

There are, however, certain advantages in using interference rather than pha contrast microscopy when the refractive index of the medium is varied, as the measurement of cell concentration. This follows from the fact that inte ference microscopy enables one to measure the o.p.d. due to the cell for an value of the refractive index of the medium, whereas phase contrast micr scopy only enables one to judge when the o.p.d. is zero. Thus, with th phase contrast microscope the cell must be immersed in a series of solutions increasing refractive index until it disappears. But, by using the interferen microscope, the refractive index of the cell (and hence the concentration) ma be simply calculated by measuring the o.p.d. caused by the cell for only tw values of the refractive index of the medium, one near that of water an another which is guessed to be somewhere near that of the cell. This calcul tion is made by using the linear relationship (equation (7)) connecting o.p due to the cell with the refractive index of the medium. The straight line co necting the two points, determined experimentally, may be extrapolated give not only the concentration but also the dry mass and the thickness the cell.

Attempts (by Barer and others, 1953) to measure the refractive index fixed cells by using the phase contrast microscope 'failed completely', since was never possible to make a fixed cell appear with reversed contrast. It w suggested that, presumably, the dead cell becomes permeable to the prote molecules in the external medium so that the external concentration nev exceeds the internal one. The refractive indices of fixed cells have be measured with the interference microscope. The results, described belo suggest that the difficulty in making cells appear with reversed contrast in t phase microscope is due to the fact that available immersion media do not ha a sufficiently high refractive index.

The fixed cell for the purpose of the following argument may be regarded a 'sack' containing particles of denatured nucleoprotein, &c., permeated spaces which may be filled with the fluid medium in which the cells are im mersed, and it is assumed that this medium does not alter, by interaction, t refractive index of the cell particles. It must also be assumed in any measu ments on fixed cells that errors arising from the heterogeneous distribution material are small. That is to say, the optical path difference due to the hete geneous cell is the same as it would be if the particles in it were packed, w no spaces, in a plate occupying the same projected area as the cell. Let μ_p the refractive index of the intracellular particles, t the geometrical thickn of the cell, and μ_m the refractive index of the medium. If it is first assum that none of the solute molecules in the medium passes into the cell but o the molecules of the solvent water of refractive index μ_w, then it can be sim shown that ϕ_m the o.p.d. due to the cell measured relative to the mediu given by

$$\phi_m = (\mu_p - \mu_m)t\,(1-f) - (\mu_m - \mu_w)tf, \qquad (7)$$

where f is the fraction of the cell volume unoccupied by particles. Hence, will become zero before μ_m attains the value μ_p.

Equation (3) now has the form

$$\chi = \frac{\frac{\mu_p - \mu_w}{m}}{At(1-f)}, \tag{11}$$

where A is the cell projected area and m the mass of material other than water in the denatured cell particles.

Hence, from equations (10) and (11), the cell dry mass m is given by

$$m = \phi_m \frac{A}{\chi} + (\mu_m - \mu_w)t. \frac{A}{\chi}, \tag{12}$$

which is identical, as is to be expected, with equation (7).

If, secondly, it is assumed that the medium permeates the spaces in the fixed cell, the measured o.p.d. ϕ_m is given by

$$\phi_m = (\mu_p - \mu_m)t\,(1-f), \tag{13}$$

and ϕ_m is zero when the refractive index of the medium is equal to that of the particles. Hence, from equations (11) and (13) the cell dry mass m is given by

$$m = \phi_m \frac{A}{\chi} + (\mu_m - \mu_w)t \frac{A}{\chi}(1-f). \tag{14}$$

It follows from either of equations (12) or (14) (i.e. whether or not solute molecules from the medium enter the cell), that there will be a linear relationship between o.p.d. ϕ_m and $\mu_m - \mu_w$.

Ram sperm fixed in 10 per cent. neutral formalin were washed several times with water and then immersed in a series of solutions of (1) bovine plasma albumen, and (2) sucrose of measured refractive index. There is some uncertainty as to whether the protein molecules from the external medium pass into the fixed ram sperm head; it seems highly likely that the small sucrose molecules will enter the cell. Since the o.p.d. varies over the sperm head, values of ϕ_m were determined at the same region in each head, the heads always being located in the same plane relative to the optic axis. The expected linear relationship between ϕ_m and $\mu_m - \mu_w$ was confirmed, the data for protein and sucrose lying in two distinct straight lines (fig. 13, A and B). This is taken to mean that whereas the sucrose molecules are able to enter the cell, the protein molecules do not enter this particular fixed cell.

From the data on sperm heads immersed in solutions of protein and sucrose, the following information has been obtained. The refractive index (μ_{mv_1}) at which the cell disappears, obtained by extrapolation, is 1·46; the geometrical thickness of the cell (from equation (12)) is 0·45 μ. The average cell concentration, i.e. the mass of dry material in the particles per unit cell volume, is given by $(\mu_{mv_1} - \mu_w)/\chi$, where χ has the value appropriate to the material in the

particles. Since, in the fixed cell, the concentration is high, the value of χ may lie between 0·19 (dilute protein solution) and 0·15 (dry protein). Since the way χ varies for desoxyribose nucleic acid (which constitutes a large fraction of the sperm head) has not yet been investigated, the above values of χ have been taken to calculate the limits for the average cell concentration, which are from 72 to 87 gm./100 c.c.

From the measurements with sucrose immersion (kindly made for us by

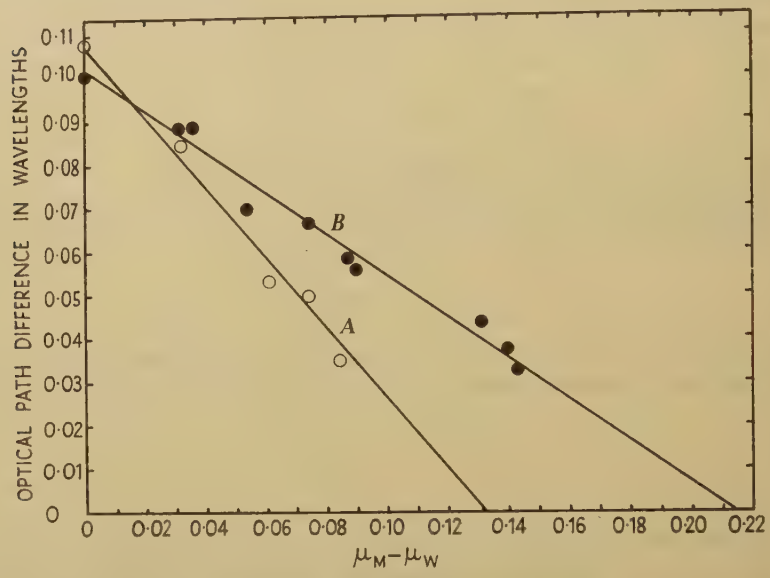


FIG. 13. Variation of o.p.d. at a chosen point in the heads of ram sperm fixed in 10 per cent neutral formalin against $\mu_m - \mu_w$, where μ_m is the refractive index of the medium in which the are immersed and μ_w the refractive index of water. *A*, sperm immersed in solutions of bovine plasma albumin; *B*, sperm immersed in solutions of sucrose.

Dr. A. J. Hale) the refractive index u_{mv_2} of the particles in the fixed cell obtained by extrapolation is 1·54; the 'effective' thickness of the cell (from equation (14)), that is the thickness of the plate in which the particles would be uniformly located, is 0·28μ. Hence the fraction $(1-f)$ of the cell occupied by particles is 62 per cent. The concentration of material other than water in the particles themselves is 118–140 gm. per 100 c.c.

The values for concentrations obtained by the above methods are expressed on a mass/volume basis and hence the maximum values which they may attain are given by the densities of the dry material. For example, the concentration for dry protein and desoxyribonucleic acids are about 127 and 165 gm./100 c.c. respectively. Clearly, if x is the concentration of dry material in the cell, then $100\rho - x$ is the concentration of water in gm. per 100 c.c., where ρ is the density of the wet material constituting the cell. The concentration of water is $100 - x$ as given by Barer and others (1953) when $\rho = 1$, and this may be a good approximation for some living cells.

Discussion of Errors and Suitability of Object for Accurate Measurement

To avoid unreliable results it is most important that measurements should be made only on objects that have suitable properties. A discussion is given below of errors of measurement and their relation to the properties of the object.

Non-constancy of χ

Inherent errors in the dry mass determinations due to the variations of χ for the different substances occurring in cells will often be small (compare table 1). However, other errors may occur in cell refractometry, and these may be compared with the errors in absorption microspectrometry which have been much discussed (see review by Davies and Walker, 1953). In absorption measurements errors may arise from failure of Beer's law, that is, from a non-linear variation of optical extinction with the concentration of material in the cells. An analogous error in cell refractometry is the variation of χ with concentration. Thus, while χ for protein solutions in concentrations up to about 0·1 gm./c.c. is constant at about 0·19, χ for a dry protein is about 0·15. Except for non-electrolytes (e.g. sugars), similar variation of χ with concentration occurs for other substances in cells. The way χ varies between dry protein and a protein concentration of 0·1 gm./c.c. needs investigation. This variation in χ may cause an apparent decrease in the dry mass of cells upon fixation, by an amount depending on the localized concentrations of material in the fixed cell, and this will partly depend upon the nature of the fixative used.

Glare

As a result of glare (due to light scattering, and reflexion by surfaces of the lenses in the microscope objective, &c.) a fraction of the light energy from each point in the object plane is randomly distributed over the image plane. In micro-absorption measurements this may result in too low a value for the optical extinction of the object and may cause serious errors at high values of optical extinction. When there are several bands in the field of view of the interference microscope, the effect of glare is to scatter some of the light energy in the bright part of the band system over the image plane so that the black bands are no longer black. The distribution of light intensity in the band system in the clear field is thus altered, but the distribution in the band system in the object is altered in a similar way. Hence the effect of glare is to reduce equally the visibility of the bands in the object and background field. This may decrease the accuracy of the measurements but will introduce no systematic error as in absorption microspectrometry.

Phase changes in the comparison beam

It may often happen that the comparison beam does not pass through a region of the slide which is entirely clear but which contains refractile structures. In the Dyson microscope the cones of light through points in the field

of view have associated with them annular comparison regions and these two regions approximately overlap. If the structures on the slide are randomly distributed along lines radiating out from the centre of the field of view, then the effect of the refractile structures in the comparison beam is merely to reduce equally the visibility of the bands in the object under examination and the surrounding clear field. No systematic error is introduced into the measurements. For example, if 30 per cent. of the area occupied by the comparison beam is obstructed by the explant in the tissue culture, then the phases of the light rays which have passed through it are effectively at random, and it can be shown that the ratio of the light intensities in the bright and dark bands is now reduced to 16:1. Measurements can still be made under these conditions.

If the objects in the comparison beam do not introduce effectively a random change of phase it may be that the average phase of this beam will alter as the slide is moved across the microscope stage, and then the bands in the field may be seen to move. Errors can always be avoided by making measurements only when the field contains a clear area (the larger the better), and the position and direction of the bands in this area can be defined. The above applies only to Dyson microscopes, where the comparison beam passes through a large area of the slide.

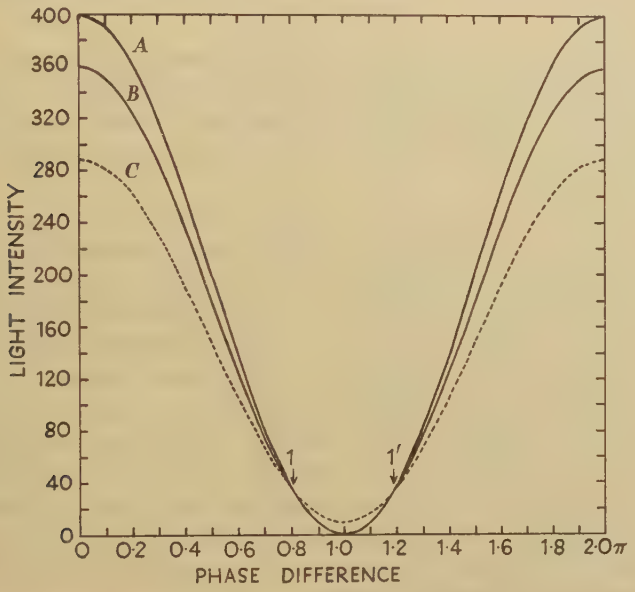
Light scatter

In absorption microspectrometry light scatter gives rise to serious difficulties, especially with ultra-violet light where the light scatter is inherently larger than it is with visible light. In cell refractometry both reflexion, refraction, or diffraction by the object which causes light to pass outside the collecting aperture of the objective, and light absorption by the object, may introduce errors whose magnitude can be roughly predicted by the following considerations. If the two interfering beams have amplitudes E_1, E_2, and phase difference θ, then the resulting amplitude E is given by the relationship

$$E^2 = E_1^2 + E_2^2 + 2E_1 E_2 \cos \theta. \qquad (15$$

The resulting intensity distribution (proportional to E^2) in the band system in the field of view is plotted (fig. 14, A) when the two interfering beams have equal intensities ($E_1^2 = E_2^2$). Figs. 14, B and C show the effect of reductions of 20 per cent. and 50 per cent. in the light intensity of one of the beams ($E_2^2 = 0{\cdot}8$ and $0{\cdot}5\ E_1^2$) corresponding to objects of optical extinction 0·1 and 0·3 respectively. These latter band systems would be seen in uniformly absorbing strip objects. Hence, neglecting any phase change, on moving a small light scattering or absorbing object across the field, the intensity in the object will differ from that in the field except at points such as 1, 1′ (fig. 14). If the optical retardation is obtained by setting the object to the position of minimum light intensity and measuring the distance from the corresponding dark band in the background field, there will be no error. However, if the densitometer trace displacement method is used there will be an error, the magnitude of which will depend on the position of the object in the band system. In living cells

ss of light due to scatter or reflexion is often negligibly small and even in xed cells visible light scatter is small compared with that found in the ultra- olet. Hence the error introduced in the o.p.d. measurement is often negligible. In order to assess the magnitude of the light loss by scatter, &c., in the easurements which have been described above, the objects were observed ithout interference contrast. This is simply achieved by putting the lower terferometer plate slightly out of adjustment. It is essential not to alter the



IG. 14. Light intensity distribution (theoretical) across the field of view of the interference icroscope for (*A*), interfering beams of intensities E_1^2, E_2^2 equal; (*B*), $E_2^2 = 0{\cdot}8\ E_1^2$ (optical extinction 0·1); (*C*), $E_2^2 = 0{\cdot}49\ E_1^2$ (optical extinction 0·3).

ocus obtained with interference contrast since the appearance of refractile bjects is known to vary with focus. Measurements on highly scattering ob- ects should be avoided.

leterogeneity in the object

An important source of error in microspectrometry results from hetero- eneous distribution of material in the cell. Similar 'distribution error' occurs n cell interferometry and, if markedly heterogeneous objects are measured, erious error may arise. When the object is granular the contrast in the inter- erence band system may be used as a rough measure of the heterogeneity; if ontrast is high the object is suitable for accurate measurement, but if the bject will not 'black-out' it is unsuitable.

Shape of object

If all parts of an irregular body can be brought into focus at the same time ccurate measurements of dry mass may be possible; if such focusing cannot

be performed errors may result. Homogeneous regularly-shaped bodies, e spheres, ellipsoids with major axis in plane of focus, &c., may give accura results even if only the central parts of the object are in focus. An example an irregular object which can only be measured roughly is the pollen grain (s section on experimental results), which contains shells and zones of markec different dry mass per unit volume.

Size of object

When the object has dimensions not much greater than the resolving pow of the microscope an accurate measure of its size and o.p.d. cannot be o tained, owing to diffraction. Hence dry mass per unit area cannot be obtain accurately, but methods are being studied for obtaining a measure of total d mass in such a body.

There is a general 'geometrical' limitation in both absorption microspectr metry and refractometry. We have seen that the dry mass and concentration bodies inside cells, e.g. the granules in microspores, can only be determin if the linear dimensions of the body and the cell are known. These data can be obtained for some cells, e.g. fibroblasts in tissue culture. These cells oft grow on the surface of the coverslip and the flattened nucleus has, above a below it, layers of cytoplasm. While it is possible from measurements in seve media of different refractive indices to measure the geometrical thickness nucleus plus cytoplasmic layers, it has not been possible to measure the thic nesses of the cytoplasmic layers themselves, although they are probably sm relative to the thickness of the nucleus. Hence, in the measurements on chi fibroblasts the dry masses were specifically referred to the nuclear project area rather than to the nucleus itself.

We wish to thank Professor J. T. Randall, F.R.S., and Dr. Honor B. Fe F.R.S., for encouragement and suggestions. We are very grateful to Mr. Dyson for the loan of his interference microscope. We also wish to thank M A. F. Huxley and Mr. F. H. Smith for discussions, Dr. P. M. B. Walker f the use of the recording densitometer, and Miss F. Ticehurst for prepari the plates. One of us (J. C.) wishes to acknowledge his gratitude to the Briti Empire Cancer Campaign for the grant which allowed him to participate this study.

Postscript (*December* 1953)

The original manuscript was sent to the Cytochemical Commissioners June 1953. Since then one of us (H. G. D.) has collaborated with Profes A. Engström and Dr. B. Lindström at the Karolinska Institute, Stockholm, a comparative determination of the dry masses of a variety of biologi structures by means of the X-ray absorption (Engström and Lindström, 194 and optical interference methods.

The results, some of which are given in the table below (Nature, 1953, **172,** 1041), show that the two methods are in good agreement.

Material		$\mu\mu$ *gm./*μ^2	
		X-rays	*Interference*
Skin (human)		..	..
Str. corneum point	1	2·7	2·5
	2	3·0	2·9
	3	3·2	2·9
	4	3·0	2·9
Str. mucosum	5	1·3	1·4
	6	0·9	1·0
	7	1·0	1·2
Aorta 1 (rabbit)		1·7–1·8	1·3
Aorta 2 (rabbit)		1·7	1·2
Gastric mucosa (dog)			
Parietal cells		0·8–1·0	0·8–1·3
Chief cells		2·3–2·6	2·2–2·8
Ventral horn cells (dog)	1	1·3	1·1
	2	1·5	1·3
	3	1·6	1·6
	4	1·2	1·3
Thyroid follicles (rat)	1	1·4	1·5
	2	1·4	1·4
	3	1·8	2·0
	4	1·8	1·7
	5	1·6	1·7
	6	1·7	1·9
	7	1·4	1·9
	8	1·5	1·9

Postscript (*June 1954*).

Messrs. Cooke, Troughton, and Simms have available a 1·3 and 0·85 N.A. Dyson microscope, which, although slightly different from the type we have described, performs in essentially the same manner. A wave-shearing type of microscope, 0·7 N.A., is available from Messrs. C. Baker.

The interferometric technique has been applied in studies of sea urchin eggs (Mitchison, J. M., and Swann, M. M., Quart. J. micr. Sci., 1953, **94,** 381); composition of bone tissue (Davies, H. G., and Engström, A., Exp. Cell Res., in press); distribution of dry mass in malignant epithelial tumours (Davies, H. G., and Engfeldt, B., Lab. Invest., in press); and in quantitative enzyme cytochemistry (Davies, H. G., Barer, R., and Danielli, J. F., Nature, in press).

A study similar to that given on p. 296 has been published by R. Barer (Nature, 1953, **172,** 1097). R. Barer and S. Tkaczyk (Nature, 1954, **173,** 821) found that χ for bovine serum albumen is constant up to a concentration of

about 50 gm. per 100 c.c. However, their statement that the refractive incre ments of sugars vary with concentration is incorrect (Davies, H. G., an Wilkins, M. H. F., Nature, 1952, **169,** 541). χ for deoxyribose nucleic aci has been given as 0·20 (wavelength 436 mμ) by T. G. Northrop, R. L. Nutte and R. L. Sinsheimer (J. Amer. Chem. Soc., 1953, **75,** 5134), and as 0·17 at 546 mμ and 0·181 at 436 mμ by M. B. M'Ewen and M. I. Pratt (unpub lished).

REFERENCES

Adair, G. S., and Robinson, M. E., 1930. Biochem J., **24,** 993.
Armstrong (Jun.), S. H., Budka, M. T. E., Morrison, K. C., and Hasson, M., 1947. Amer. chem. Soc., **69,** 1747.
—— 1953. Ibid., **172,** 1097.
Barer, R., 1952. Nature Lond., **169,** 366.
—— Ross, K. F. A., and Tkaczyk, S., 1953. Ibid., **171,** 720.
Bawden, F. C., and Pirie, N. W., 1937. Proc. Roy. Soc. B, **123,** 274.
Bernal, J., and Fankuchen, I., 1941. J. gen. Physiol., **25,** 111.
Chayen, J., Davies, H. G., and Miles, U. J., 1953. Proc. Roy. Soc. B, **141,** 190.
Davidson, J. N., and Leslie, I., 1950. Cancer Res., **10,** 587.
Davies, H. G., and Walker, P. M. B., 1953. *Progress in biophysics*, ed. Butler, J. A. V., an Randall, J. T. London (Pergamon Press), **3,** 195.
Davies, H. G., and Wilkins, M. H. F. 'Cytochemistry Commission of the Soc. for Ce Biol.', *Physical aspects of cytochemical methods*, Stockholm, Sept. 1951.
—— —— 1952. Nature Lond., **169,** 541.
—— —— (Unpublished.)
Dyson, J., 1949. Proc. Phys. Soc. B, **62,** 565.
—— 1950. Ibid., A, **204,** 170.
—— 1953. Nature Lond., **171,** 743.
Engström, A., and Lindström, B., 1949. Ibid., **163,** 563.
Handbook of chemistry and physics. Ohio (Rubber Co.).
Huxley, A. F., 1952. J. Physiol., **117,** 52 P.
Jenkins, F. A., and White, H. E., 1951. *Fundamentals of optics*. New York (McGraw-Hi
La Cour, L. F., 1949. Heredity, **3,** 319.
Lebedeff, A. A., 1930. Rev. d'Optique, **27,** 385.
Mann, T., 1949. Advances in Enzymology, **9,** 329.
Oettlé, A. G., 1950. J. Roy. micr. Soc., **70,** 232.
Ogur, M., Erikson, R. O., Rosen, G. V., Sax, K. B., and Holden, C., 1951. Exp. cell Re **2,** 73.
Oster, G. (Private communication.)
Painter, T. S., 1943. Bot. Gaz., **105,** 58.
Perlman, G. E., and Longsworth, L. G., 1948. J. Amer. chem Soc., **70,** 2719.
Sax, K., and Edmonds, H. W., 1933. Bot. Gaz., **94,** 156.
Smith, F. H., 1950. British Patent Spec. No. 639014.
Tennent, H. G., and Villbrandt, C. F., 1943. J. Amer. chem Soc., **65,** 424.
Uber, F. M., 1939. Amer. J. Bot., **26,** 799.
Vendrely, R., and Vendrely, C., 1949. Experientia, **5,** 327.
Walker, P. M. B., 1953. J. Sci. Instr., **30,** 162.
—— and Yates, H. B., 1952. Proc. Roy. Soc. B, **140,** 274.
Williams, W. E., 1948. *Applications of interferometry* (Methuen & Co. Ltd.).

This paper is issued under the auspices of the Cyochemtistry Commission.

Cytological Studies of the Neurones of *Locusta migratoria*

Part II. Cytoplasmic inclusions during the differentiation and growth of the nerve cells

By SAIYID AHMAD SHAFIQ

(*From the Cytological Laboratory of the Department of Zoology and Comparative Anatomy, and the Hope Department of Entomology, Oxford*)

With one plate (fig. 1)

Summary

1. The Golgi controversy as it applies to the nerve cells of *Locusta* is discussed with reference to the recent publications of Beams and others (1953) and Gatenby and others (1953). Further support has been obtained for the view that the 'Golgi' appearances are produced by reactions at the surface of the lipochondria.

2. Cytoplasmic inclusions during the differentiation and growth of the nerve cells are described. The early germ-band cells have two categories of inclusions—*mitochondria* (granular and filamentous) and *lipochondria* (osmiophil bodies). The neuroblasts and early ganglion-cells possess only granular mitochondria. The filamentous mitochondria and lipochondria appear again in the growing neurones, so that at the time of hatching of the embryo the nerve cells have attained the full development of their cytoplasmic inclusions. Thus, Gatenby's three-phase theory for nerve cells does not apply to *Locusta*.

3. It is concluded that the lipochondria and filamentous mitochondria are not necessarily self-reproducing bodies.

4. Masson's technique shows granules staining red with acid fuchsin in the growing neurones as well as in the different stages of the adult. These are shown to be lipochondria. No secretory cycle could be detected.

Introduction

THE cytoplasmic inclusions of the motor neurones of the thoracic ganglia of the adult *Locusta migratoria* were described in an earlier paper (Shafiq, 1953). Almost simultaneously Beams, Sedar, and Evans (1953) published their work on the cytology of the nerve cells of certain grasshoppers, and Gatenby, Moussa, Elbanhawy, and Gornall (1953) reviewed the Golgi controversy in nerve cells in general. The work of these authors and of Dornesco (1934) and Muliyil (1935) represents the classical views about the 'Golgi component' of the nerve cells of insects, and it will be desirable to give a summary of their conclusions here, so that they may be discussed later on with reference to the present work.

The opinions of Beams and his associates (1932, 1953) about the neurones of the grasshopper are that the spheroids seen after neutral red staining are new formations consisting of aggregated dye particles, and that the Golgi

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 305-314, Sept. 1954]

element of the neurones consists of curved and circular dictyosomes only. The osmiophobe portion described earlier (Beams and King, 1932) is an optical artifact, as it is not seen by the electron microscope.

Dornesco (1934) from his studies of the neurones of dragonflies disagreed with the above authors, for he did not regard the neutral red bodies as artifacts. He considered that while these bodies existed in life, they could not be impregnated by Golgi techniques.

Muliyil (1935) repeated the work on the neurones of the Orthoptera. He studied living cells and ultracentrifuged ganglia and concluded that the neutral red bodies existed in life and could also be impregnated by Da Fano's technique; but he regarded them as different from the Golgi bodies.

Gatenby and others (1953) also believe that the Golgi apparatus (dictyosomes) of the invertebrate neurones is different from the neutral red bodies or vacuome granules, and that the latter correspond to the 'senility pigment' of vertebrate neurones.

Material

Cells were studied during the various embryonic stages, in the nymphal instars, and at different ages of the adult of *L. migratoria*. Specimens were kindly supplied by the Anti-locust Research Centre, London.

The following information on the embryonic stages of *Locusta* is taken from the studies of Roonwal (1936, 1937). The data on the nymphal and adult stages were given by Mr. P. Hunter Jones of the Anti-locust Research Centre, London.

The cleavage cells produced by the divisions of the zygote nucleus migrate to the periphery, especially to the posterior end of the egg, to form the germ band at the age of about 28 hours. This becomes divided into the primary head region and the primary trunk region at the age of 42 hours. At the same time an inner layer of cells differentiates, so that the embryo becomes two-layered having the outer ectoderm and the inner layer (mesoderm). Neuroblasts differentiate in the ectoderm of the head region at the age of 59 hours and in the trunk region at $65\frac{1}{2}$ hours. They produce columns of ganglion cells by repeated unequal mitoses. The small cells budded off from the neuroblasts become concentrated into segmental ganglia at the age of 112 hours. The ganglia separate from the underlying dermatogenic tissue and the cells produce nerve fibres, thus attaining their definitive form. Neuroblasts become indistinguishable on the seventh day and ganglion cells begin to grow. The embryo hatches on the thirteenth day. The stock at the Anti-locust Research Centre, however, hatches in 8–10 days at 32° C. After hatching the locusts go through five nymphal instars. If they are kept at about 28° C. at night and about 35° C. during the day, the total duration of the nymphal period is about 20 days, after which the adults emerge. The newly emerged adults are grey but when they mature in about 4 to 6 weeks' time, the males become yellow and the females brown. The total adult life is about 3 months.

Methods

The methods used in this study of the cells from embryonic and nymphal stages are similar to those used in the work on adult neurones. They may be enumerated as follows:

(1) study of living cells by phase-contrast microscopy;
(2) vital staining by neutral red, Janus green, and Janus black;
(3) Baker's (1944) Sudan black method for 'Golgi' component;
(4) Baker's (1946) acid haematein test for phospholipines;
(5) Sudan black method on paraffin sections. For this, ganglia were fixed in Helly's fluid (Thomas, 1949) and in Champy's fluid (Baker, to be published shortly);
(6) Regaud's (1910) method for staining mitochondria;
(7) Metzner's method for staining mitochondria (Metzner and Krause, 1928), with Altmann's and Helly's fluids as fixatives;
(8) osmium-impregnation technique of Kolatchev (1916), with Meves's and Champy's fluids as fixatives;
(9) silver-impregnation technique of Aoyama (1929);
(10) Masson's tricolor staining method.

The cutting of paraffin sections was easiest when the embryos had been dissected away from the yolk. This, however, could not be done with late embryos, where the yolk is enclosed in the gut, and therefore various softening procedures were tried. Successful preparations could be made when cedarwood oil was used as the antemedium for paraffin embedding; this was followed by soaking the blocks in Baker's (1941) softening mixture. Phenol-xylene as antemedium for paraffin embedding, with soaking of the blocks in water, also gave some successful preparations.

Observations

The early cleavage cells were studied in the living state by phase-contrast, and they appeared to possess filamentous and granular mitochondria and spheroid bodies. The mitochondria could be stained by the Janus dyes and the spheroids by neutral red, but attempts to study these inclusions in fixed preparations were not successful; so these cells will not be described here.

When the cleavage cells have formed the germ-band, the cells are arranged in several layers. These cells are cuboidal in shape and are about 26μ long, their nuclei being 13μ in diameter. The germ-band was dissected out from the eggs and its cells could easily be studied in the fixed preparations as well as in the living state. They have the following inclusions in their cytoplasm.

Mitochondria

The mitochondria of these cells are of two types, granular and filamentous. The granular type are about 0·5μ in diameter and are more numerous than the

filamentous, which are thin threads about 1·2μ long. They were seen by phase-contrast and could be stained by Janus black supra-vitally. Fixed preparations by the method of Regaud also showed them. They are evenly distributed throughout the cytoplasm.

Lipochondria (osmiophil bodies)

Dispersed among the mitochondria are seen spheroids of diameters varying from 0·7μ to 1·2μ (fig. 1, C). Neutral red stained these bodies when the germ-band was immersed in a 0·01 per cent. solution of the dye, dissolved in saline, for about 15 minutes. By positive phase-contrast they sometimes appear to be binary in structure, having an outer dark cortex and a lighter inner medulla (fig. 1, C); but as with the lipochondria of the nerve cells, it is not possible to assert definitely whether this is an optical illusion or not. They can be impregnated by Kolatchev's method for the Golgi apparatus (fig. 1, E). In the figure some of these spheroids are over-impregnated and appear like dense granules, while on others the osmium has been deposited on the surface only.

After this early germ-band stage, the cells greatly increase in number and the germinal layers are formed. The individual cells are now much smaller with little cytoplasm, and are difficult material for the study of their cytoplasmic inclusions.

The embryos become very suitable for study from the stage when the neuroblasts differentiate and thereafter. They are now sufficiently big to be dissected out of the egg and manipulated in various ways. Neuroblasts of the head as well as those of the trunk region were studied; no differences could be seen between them. They are big cells measuring about 35μ in diameter, their nuclei being 18μ in diameter, so that there is a large amount of cytoplasm in the cells. Phase-contrast microscopy shows only one type of granule in the cytoplasm (fig. 1, D). These granules are spheroidal, measuring 0·6μ in diameter, and are uniformly distributed throughout the cell. They appear dark

---

FIG. 1 (plate). All photomicrographs are at the same magnification.

A, a motor neurone from the adult locust, to show granules staining with acid fuchsin. The ganglion was fixed in Helly's fluid and 6μ paraffin sections were stained by Masson's tri-color stain.

B, a motor neurone from the adult locust stained as A, after the ganglion had been extracted with Baker's Bouin-pyridine method.

C, cells from the early germ-band of the locust embryo as seen by phase-contrast microscopy. Spheroids and mitochondria are seen.

D, a neuroblast (in anaphase) as seen by phase-contrast. The granular mitochondria are uniformly distributed throughout the cytoplasm; lipochondria are absent.

E, cells of the early germ-band prepared by Kolatchev's method for the Golgi apparatus. The embryo was fixed in Champy's fluid and osmicated for 4 days at 37° C.; 6μ section.

F, neuroblast and the ganglion-cells produced by the division of the neuroblast; prepared by Aoyama's method for the Golgi apparatus.

G, embryonic neurones as seen in a Sudan black preparation. The embryo was fixed in Helly's fluid and embedded in paraffin.

H, embryonic neurone of a slightly earlier stage than in G, prepared by Aoyama's method for the Golgi apparatus.

I, neurones from an embryo about to hatch. Prepared by the same method as E, except that Meves's fluid was used as fixative.

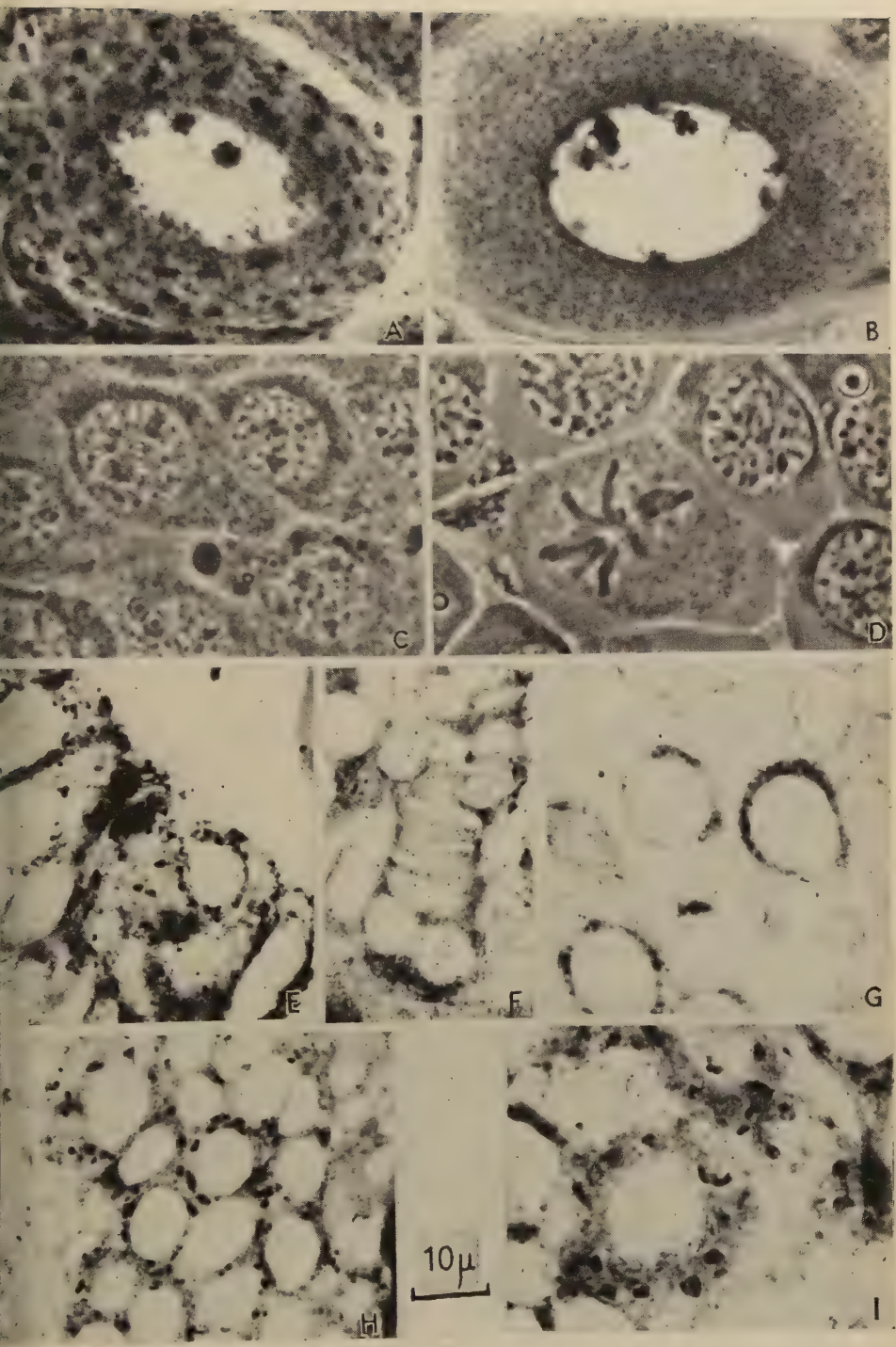


FIG. 1

S. A. SHAFIQ

by positive phase-contrast. They stain strongly by Janus green and Janus black. Neutral red does not stain anything in the cells except in very early neuroblasts, when sometimes one or two granules were seen after staining the cells supravitally. Regaud's method for mitochondria shows the granules clearly. Sudan black stains them in frozen gelatin sections and also in material fixed in Helly's fluid and embedded in paraffin. They are also positive for the acid haematein test for phospholipines. The impregnation techniques of Aoyama and Kolatchev do not show any trace of 'dictyosomes' or 'platelets', but only small granules (fig. 1, F). They correspond to the granules seen in the living cell and probably represent them.

It is, therefore, concluded that there are no lipochondria in the neuroblasts and that the granules seen in the cytoplasm of the neuroblast are mitochondria. Further, the histochemically demonstrable lipide content of the cell is a constituent of the mitochondrion.

The columns of the ganglion cells produced by the division of the neuroblast were studied. Each cell is 13μ in diameter with a nucleus 10μ in diameter. In their cytoplasm also only one type of granules, the mitochondria, could be made out. The impregnation techniques show no 'platelets' or 'dictyosomes' (fig. 1, F); the young ganglion cells resemble the neuroblast in this respect.

When the ganglion cells have produced fibres, concentrated themselves into segmental ganglia, and begun growing, they present a different picture from the neuroblasts and the early ganglion cells. Treatment with Sudan black now reveals lipochondria of diameters up to 0·9μ in the cells (fig. 1, G). 'Golgi' appearances could also be produced at this stage (fig. 1, H) by deposits on the surface of the lipochondria. These first-formed lipochondria are smaller than the lipochondria of later stages (where their diameters vary from 0·4μ to 2·6μ). They do not arise from any special region of the cytoplasm, but from the beginning of their appearance they are uniformly distributed throughout the cytoplasm, like the mitochondria. It is thus not possible to say whether the lipochondria arise anew in the cytoplasm or are formed by the transformation of granular mitochondria.

During the later stages of the embryo the lipochondria grow and some of them have attained almost the same size as that of the larger lipochondria in the adult neurone. These give the characteristic appearance of 'curved and circular dictyosomes' or 'osmiophil platelets', by Golgi techniques, and even in size they are comparable to the platelets of the adult neurone.

The only difference between the lipochondria of the adult and the embryonic neurones that could be found is that the lipochondria of embryonic neurones are probably more readily coloured by neutral red than those of the adult.

The mitochondria were also studied in these late embryos. In the living nerve cells the granular as well as the filamentous mitochondria are easily seen by phase-contrast; they also stain by Janus black supravitally. In the fixed preparations they were seen by Metzner's method for mitochondria. It is thus obvious that at this stage the nerve cells in *Locusta* are fully differentiated

from the point of view of their cytoplasmic inclusions, their cytological picture being essentially the same as that of the neurones of the adult.

The lipochondria were also studied in the various instars of the locust and in immature, mature, and old locusts. For this study material was fixed in Helly's fluid and embedded in paraffin. Sections were stained by Sudan black. Living cells were also studied. The neurones of the thoracic ganglia were

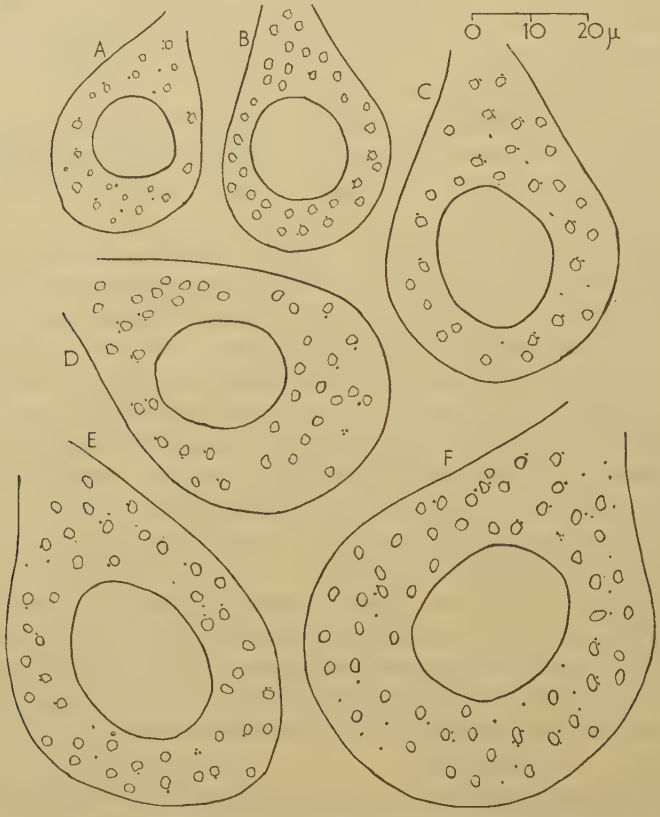


FIG. 2. Large neurones of locusts at various stages in development. The ganglia were fixed in Helly's fluid and embedded in paraffin by Thomas's method; sections were coloured by Sudan black. All the figures are camera lucida drawings at the same magnification. A, neurone from 1st instar nymph. B, from 2nd instar nymph. C, from 3rd instar nymph. D, from 4th instar nymph. E, from 5th instar nymph. F, from an adult locust.

chosen for study. They vary greatly in size in all the different instars, as they do in the adult. Thus it is possible to find some neurones in the ganglia of the first instar which are bigger than the smaller neurones of the adult locust. But taking the cell population of the ganglion as a whole, the neurones obviously increase greatly in size. With the increase in the size of the neurones from the first instar to the adult stage there is correspondingly a great increase in the number of the lipochondria. The situation is depicted in the series of camera lucida drawings of the larger neurones from the different instars of the locust

g. 2). The drawings were all made from the material fixed in Helly's fluid d embedded in paraffin. A definite quantitative relationship between the mber of the lipochondria and the size of the neurone could not be obtained cause of the difficulty of correctly estimating the number of lipochondria of fferent sizes in the cells. The main conclusion derived from a general comrison of the neurones in the various instars is that during the growth of the urone in the different nymphal instars the lipochondria increase in number, eir sizes remaining more or less the same. This is clearly seen from the mera lucida drawings.

The lipochondria in immature, mature, and old locusts were also compared d no differences were found.

eurosecretion granules

Wigglesworth (1950) remarked about neurosecretion, 'It is characteristic of urosecretory cells that they contain droplets of colloid substance which ains with acid fuchsin. Cells of this type are found in the "pars intercerebralis" or medial dorsal region of the brain, in the corpus cardiacum and in rious ganglia of the nerve cord.' Thomas made a study of neurosecretion ith reference to the cytology of nerve cells in molluscs and vertebrates and ut forward the view (1951) that the intraneuronal granules or the neurocretion granules are formed in the 'spheroids' (lipochondria).

Thoracic ganglia of immature, mature, and old locusts of both sexes and so the thoracic ganglia of the various nymphal instars were studied by lasson's tricolor stain. Granules staining red with acid fuchsin were seen in ost of the neurones of all the stages mentioned above. Their number, size, d distribution leaves no doubt that it is the lipochondria that are being ained by acid fuchsin (fig. 1, A). Lipides were extracted from some ganglia y Baker's Bouin-pyridine method (Baker, 1946). After this treatment the haracteristic lipochondria were lost, and Sudan black showed only fine granes in the section. Masson's stain also showed only fine granules in these ctions (fig. 1, B). This is thus further evidence that in *Locusta* the acid chsin in Masson's method stains the lipochondria of various sizes.

It is important to mention, however, that though acid fuchsin stained the pochondria more strongly in some cells than in others, a secretory cycle as escribed by Scharrer (1941) in the neurosecretory cells of the cockroach *eucophaea* was not observed. Beams and King and Muliyil also did not find secretory cycle of 'Golgi bodies' in the Orthoptera they studied.

Conclusions

ipochondria and 'Golgi bodies'

The classical views on the Golgi problem in insect nerve cells have been mmarized in the introduction. The usual conclusion is that there are three pes of inclusions in insect nerve cells—the mitochondria, the 'neutral red'

or 'vacuome' granules, and the Golgi 'dictyosomes'. However, it was clear shown in the earlier study (Shafiq, 1953) that neutral red stained th lipochondria and that the impregnation techniques produced the Gol appearance in or on the lipochondria. The present work provides furth evidence in support of this view. Thus, when the lipochondria are presen as in the neurones of the various nymphal instars and in various stag of the adult, the Golgi appearances can be produced on them. When, the growing embryonic neurones, the lipochondria are smaller than in lat stages, the dictyosomes formed by Golgi techniques are also small. Ar finally, when the lipochondria are absent, that is, in the neuroblasts and ear ganglion cells, dictyosomes cannot be produced by any impregnation tec niques.

Some comments can be made now on the various classical works. Thus it t interesting to see that in Muliyil's experiments (1935) with the ultracentrifug the neutral red granules (smaller lipochondria) and 'Golgi bodies' (i.e. artifac on the surfaces of larger lipochondria) collected at the same (centripetal) po and remained intermingled with each other.

Gatenby and others (1953) would homologize the senility pigment gra ules of the neurones of vertebrates with the 'neutral red granules' of th neurones of invertebrates. Now, the neurones of old insects sometimes conta granules of yellow pigment, and this may perhaps be formed by a modificati of the contents of the lipochondria ('neutral red granules'). However, the tr homologue of the insect's lipochondria are the colourless lipochondria of th neurones of vertebrates (the 'spheroids' of Thomas (1951)).

Beams and others (1953) impregnated the ganglia with osmium by Kola chev's technique and studied the deposits of osmium by the electron microscop They say that 'what we have described here as Golgi bodies are not gross ar facts' and that 'the Golgi bodies are relatively opaque to the electrons but th do not seem to be completely homogeneous as is evidenced by the light appearing areas within them'. It seems doubtful whether any useful purpo is served by making an osmium deposit on the surface of a cytoplasmic i clusion and then examining the form of that deposit with the electron micr scope.

It is a pity that the methods of research developed and recommended in t pioneer studies of Baker (1944, 1946) are not applied by more workers, befo drawing conclusions about the 'Golgi apparatus'. When these methods a applied to the insect nerve cells it is obvious that neutral red stains the lip chondria and that impregnation techniques produce 'dictyosome' appearanc on the lipochondria.

Origin of cytoplasmic inclusions

Gatenby (1919) and Hirschler (1918) studied the early development of t gastropod *Limnaea* by Golgi techniques. They found that in the cleaving e the mitochondria and 'Golgi bodies' are equally divided between the daugh cells. Gatenby and Hirschler therefore conclude that the 'Golgi bodies' a

f-reproducing bodies and as Gatenby said '. . . are able to assimilate, grow d divide in the cytoplasm somewhat as a protist assimilates, grows and ides in its watery medium'.

This study, however, leads to a different conclusion. Osmiophil bodies are esent in the earliest stages in the *Locusta*, as in *Limnaea*, but they disappear the neuroblasts. In living cells and also in impregnated sections, nothing is be seen in the cytoplasm except mitochondria. Osmiophil bodies appear ain in the growing neurones. And when these characteristic lipochondria ve made their appearance, the 'Golgi dictyosomes' can also be produced by pregnation techniques. These appearances cannot be produced in the uroblast or the early ganglion cells. Gatenby and Hirschler did not study e organogeny of any tissue but studied only the very early stages and lult tissues. It would appear that they were wrong in their conclusion. The miophil bodies are not continuously self-reproducing organelles. They can sappear and reappear. It is not possible to say whether they appear indepen-ntly in the cytoplasm, or are derived by transformation of the mitochondria. The same conclusion may possibly be applicable to the mitochondria. here are no filamentous mitochondria in the neuroblasts and early ganglion-lls, though they are present in the early germ-band cells and in the neurones the late embryos. Bensley (1953) does not regard the mitochondria as finite organelles and has shown that the mitochondrial substance is expend-ble. Harvey (1946) and Gustafson (1953) hold the same view. There is no ubt in the present study that the filamentous mitochondria disappear and appear. It is not certain whether they originate from granular mitochondria.

ecretion and neurosecretion

Nerve cells in the various growth stages were studied by Rau and Ludford 925) in the chick and by Gatenby and others (1953) in Amphibia. On the asis of this work, Gatenby and others divide the life of the neurone into an rly phase, a middle or secretory phase, and a regressive phase. They consider at the neurones are not fully developed cytologically in the early phase when e neurones function as a nervous unit only, that the Golgi apparatus is fully rmed only in the secretory phase, and that it degenerates into vacuoles and tty globules in the regressive phase.

In *Locusta* the neurones have attained full cytological development before e embryos hatch. A secretory cycle was not seen in the cells, and as regards e lipochondria no appreciable difference is noted from the embryonic stages, rough the various instars, up to the old locusts. Thus this three-phase theory oes not apply to *Locusta*.

Gatenby, as was noted above, regards secretion as a general property of erve cells. Many other workers hold the same view. The Scharrers (1953), owever, think that neurosecretion is not a general property of nerve cells and egard neurosecretory cells as a distinct cell type. Dr. K. K. Nayar (personal ommunication) regards the larger neurones of *Locusta* (diameter about 90μ)

as neurosecretory. If they are neurosecretory they appear to differ from ot nerve cells only in size. Neurosecretion certainly needs careful definiti B. Scharrer is going to undertake this (personal communication.)

The work was done under the supervision of Dr. J. R. Baker and I am v grateful to him for suggesting the problem and guiding me all through work. I am also thankful to Professor A. C. Hardy for accommodating me the Department of Zoology and Comparative Anatomy at Oxford and to I B. M. Hobby for much helpful advice.

The work was done during the author's term of Rhodes Scholarship a study-leave from Dacca University, Pakistan.

REFERENCES

Aoyama, F., 1929. Zeit. wiss. Mikr., **46**, 489.
Baker, J. R., 1941. J. Roy. micr. Soc., **59**, 75.
—— 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— To be published.
Beams, H. W., and King, R. L., 1932. Journ. Morph., **53**, 59.
Beams, H. W., Sedar, A. W., and Evans, T. C., 1953. La Cellule, **55**, 291.
Bensley, R. R., 1953. J. Histochem. and Cytochem., **1**, 179.
Dornesco, G. T., 1934. Bull. Hist. Appl., **11**, 5.
Gatenby, J. B., 1919. Quart. J. micr., Sci., **63**, 445.
Gatenby, J. B., Moussa, T. A., Elbanhawy, M., and Gornall, J. I. K., 1953. La Cell **55**, 137.
Gustafson, T., 1953. J. Embryol. and exp. Morph., **1**, 251.
Harvey, E. B., 1946. J. exp. Zool., **102**, 253.
Hirschler, J., 1918. Arch. f. Mikr. Anat., **91**, 140.
Kolatchev, A., 1916. Arch. russes d'anat. d'hist. d'emb., **1**, 383.
Metzner, R., and Krause, R., 1928. In Aberhalden's *Handbuch der biologischen Arbe methoden*, Abt. V, 2: 1, 325.
Muliyil, J. A., 1935. Zeit. Zellforsch. Mikr. Anat., **23**, 627.
Nayar, K. K., personal communication.
Rau, A. S. and Ludford, R. J., 1925. Quart. J. micr. Sci., **69**, 509.
Regaud, C., 1910. Arch. d'Anat. Mikr., **11**, 291.
Roonwal, M. L., 1936. Phil. Trans. Roy. Soc. B, **226**, 391.
—— 1937. Ibid., **227**, 175.
Shafiq, S. A., 1953. Quart. J. micr. Sci., **94**, 319.
Sharrer, B., 1941. J. Comp. Neur., **74**, 93.
—— Personal communication.
Sharrer, E., and Sharrer, B., 1953. Science, **118**, 579.
Thomas, O. L., 1949. Stain. Tech., **24**, 201.
—— 1951. J. Comp. Neur., **95**, 73.
Wigglesworth, V. B., 1950. *The principles of insect physiology*. London (Methuen & C

tological Studies of the Neurones of *Locusta migratoria*

art III. Histochemical Investigations, with Special Reference to the Lipochondria

By SAIYID AHMAD SHAFIQ AND W. G. BRUCE CASSELMAN

om the Cytological Laboratory, Department of Zoology and Comparative Anatomy, Oxford)

With one plate (fig. 1)

Summary

The motor neurones of the thoracic ganglia of adult *Locusta migratoria* have been idied by *in situ* histochemical techniques. Phospholipides and unsaturated cerebro- es are concentrated in the lipochondria; acetalphosphatides, cholesterol, protein, and onucleic acid are diffusely present throughout the cytoplasm; little nucleic acid is monstrable in the nuclei.

Introduction

N earlier studies of the neurones of *Locusta migratoria* (Shafiq, 1953, 1954) it was found that these cells contain lipochondria (osmiophil bodies). It as concluded that the 'neutral red' or 'vacuome' granules are these structures ained by such dyes as neutral red or methylene blue; that the 'Golgi' pictures 'curved and circular dictyosomes' are produced by deposits of osmium or ver on the lipochondria; and that the 'secretion' or 'intraneuronal' granules aining with acid fuchsin in paraffin sections are in fact lipochondria. The esent paper deals with the histochemistry of these nerve cells with special ference to the lipochondria. Such an investigation of these cells does not pear to have been reported previously.

Material and Methods

The motor neurones of the thoracic ganglia of mature, adult *L. migratoria* ere studied. The insects were kindly supplied by the Anti-locust Research entre, London. The ganglia were dissected out and fixed in formaldehyde- lcium (Baker, 1944), cobalt-formaldehyde-calcium (McManus, 1946*b*), or rra's (1946), Helly's, or Zenker's fluid. Some ganglia fixed in formaldehyde- lcium were postchromed in 5 per cent. potassium dichromate for 16–18 urs at 37° C. and others were treated overnight with 0·1 per cent. 'Aerosol T' (di-octyl ester of sodium sulphosuccinic acid, British Drug Houses Ltd., w called 'Manoxol OT'). Frozen sections of gelatin-embedded ganglia or araffin sections were cut as required. Some fresh, unfixed ganglia were ex- acted with cold or hot acetone or hot ether by Keilig's (1944) method as odified by Pearse (1953). Other ganglia were fixed in weak Bouin's fluid and tracted with pyridine (Baker, 1946).

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 315-320, Sept. 1954.]

For the detection of lipides Sudan black B was most often used as a saturat solution in either 70 per cent. ethanol (Baker, 1944) or propylene gly (Chiffelle and Putt, 1951). The reagents were allowed to act for as long 30 minutes at room temperature, 37° C. or 60° C. Additional studies w made with saturated solutions of acetylated Sudan black B (Lillie and Burtn 1953; Casselman, 1954) or of Sudan IV in 70 per cent. ethanol or of red O in *iso*-propyl alcohol (Lillie, 1944), and with 0·02 per cent. and 1 p cent. aqueous Nile blue sulphate (Cain, 1947*a*, 1948). The acid haemat test was chosen for the detection of phospholipides and phosphatidic ac (Baker, 1946, 1947*b*; Cain, 1947*b*; Casselman, 1952). For acetal-phosphatic Hayes's (1949) and Cain's (1949) variants of the plasmal reaction, with ad tional tests recommended by Danielli (1953), were employed. A modifi Liebermann–Burchardt reaction (Romieu, 1925) was used for detecti cholesterol and its esters.

A number of investigations were made with the periodic acid Sch (McManus, 1946*a*) and performic acid Schiff (Lillie, 1952) tests. Cont sections were either acetylated with acetic anhydride in glacial acetic a (Casselman, Macrae, and Simmons, 1954) or treated with bromine gas o saturated aqueous solution of bromine. The Schiff's reagent for these a other tests requiring it was prepared as described by de Tomasi (1936).

The distribution of pentosenucleic acids was studied in paraffin section tissues fixed in Helly's, Serra's, or Zenker's fluid by using toluidine blue pyronin/methyl green (Brachet, 1953; Jordan and Baker, unpublished). So sections were first treated with 1 *N*. HCl (Dempsey, Singer, and Wisloc 1950) or 4 per cent. trichloroacetic acid (Schneider, 1945). The nucleal action (Feulgen and Rossenbeck, 1924) was used for the study of deoxypento nucleic acids.

Tests for proteins included the coupled tetrazonium reaction introduced Danielli (1947, 1949) and further developed by Pearse (1953), and Millo (Bensley and Gersh, 1933), Sakaguchi's (Baker, 1947*a*), and Chèvremont a Frederic's (1943) tests.

Observations and Discussion

The various tests were applied first to unextracted tissues and then extracted ones.

Unextracted neurones

In frozen sections of ganglia fixed in formaldehyde-calcium or coba formaldehyde-calcium the lipochondria of the neurones are coloured intens by Sudan black B, while the cytoplasm is only weakly Sudanophil. Strong coloration is obtained when this reagent in propylene glycol is used for minutes at 60° C. Unlike those in other species (Baker, 1949) these lipocho dria can be coloured readily without postchroming the tissue, although t does improve the results.

After 'Aerosol' treatment the lipochondria are no longer discernible but the oplasm of the motor neurones is diffusely and quite strongly Sudanophil. At least some of the lipochondria resist paraffin embedding, regardless of fixative used. They are best preserved when fixed in Helly's or Champy's id or when postchromed after formaldehyde fixation. When fixed in Bouin's id they are much reduced in size.

The lipochondria are coloured as intensely by acetylated Sudan black B as the unacetylated, but whereas the acetylated derivative is readily extracted 70 per cent. ethanol, the Sudan black B is not extracted even after 20 nutes. In this case, the 'stable Sudanophilia' is due to lipide material, be-use it is not demonstrable in ganglia which have been extracted with pyri-ne. Similarly, Sudan black B was not appreciably extracted after 35 minutes' eatment with 70 per cent. ethanol from highly purified lecithins tested on garette paper (Baker, 1946) or by Coujard's (1943) technique. The pheno-enon of 'stable Sudanophilia' was first described by Lillie and Burtner (1953) their studies of human neutrophil leucocytes. They attribute it to a emical reaction between Sudan black B and some tissue-constituent rather an to the ordinary solution of the colorant in lipide.

When frozen sections are treated with oil red O or Sudan IV for 5 or 30 inutes at room temperature or at 60° C., nothing within the motor neurones coloured, but the fat-body surrounding the ganglia gives an intensely ositive reaction.

Diffuse blue staining throughout the cells results when the Nile blue test applied to frozen sections of formaldehyde-calcium-fixed tissues, whether ostchromed or not. The lipochondria cannot be distinguished.

Some tests were carried out on pairs of frozen sections. One section re-ained in distilled water and served as a control, while the other was extracted ith cold acetone for 24 hours. After this, they were simultaneously treated ith Sudan black B. There are no differences between the coloration of the pochondria or cytoplasm in the two sections, even though the reaction of the rrounding adipose tissue is considerably reduced by the acetone extraction. Thus, there appears to be little or no triglyceride present in the lipochondria r the cytoplasm of the motor neurones. Acetone extraction does not decrease eir coloration by Sudan black B. They do not give positive reactions with oil ed O, Sudan IV, or the oxazone of Nile blue. On the other hand, the presence f compound lipides is suggested by the resistance of the lipochondria to araffin embedding and their behaviour with Sudan black B. This was verified y further studies.

The acid haematein test gives a strong positive reaction with the lipo-hondria and a weak diffuse reaction in the cytoplasm (fig. 1, A). This is bsent from the pyridine-extracted controls (fig. 1, C) and is not obtained if he postchroming is omitted. The reaction is, therefore, a true positive one, pecific for phospholipides and phosphatidic acids, and is not due to other rganic substances which yield false positive reactions or to inorganic con-tituents acting as mordants for the haematein.

With the Liebermann–Burchardt reaction, a diffuse, weakly positive action is obtained which does not differentiate between the cytoplasm a the lipochondria.

The cytoplasm of the neurones also gives a diffuse weak reaction w Schiff's reagent after treatment with mercuric chloride (Cain, 1949; Hay 1949) or 0·1 *N.* HCl (Danielli, 1953). Neither unfixed nor formaldehyc calcium-fixed ganglia react directly with Schiff's reagent. Therefore, neitl free aldehydes nor any carbonyl compounds resulting from atmosphe oxidation or the action of formaldehyde on unsaturated lipides (Wolman a Greco, 1952) are present. The positive plasmal reaction may thus be attribut to acetalphosphatides and is not a pseudo-plasmal reaction.

In paraffin sections of Helly- or Zenker-fixed ganglia and in frozen sectic of formaldehyde-calcium-fixed ones the lipochondria give positive reactic and the cytoplasm weak diffuse ones with the PAS (fig. 1, D) and PFAS tes The PAS reactions are blocked by acetylation and the PFAS ones by bromir tion. Both remain positive when applied to tissues in which aldehydes produc by the plasmal reaction are first blocked by sodium bisulphite.

With toluidine blue or the pyronin/methyl green reagent the cytoplasm the neurones is strongly basiphil. The reactions are greatly reduced by fi treating with HCl or trichloracetic acid, suggesting that the basiphilia is d to pentosenucleic acids. In the motor neurones the nucleal reaction is negati although it is positive in the nuclei of the smaller cells of the ganglia. Neith of the nucleic acids could be localized in the lipochondria.

The various protein tests give diffuse positive reactions throughout t cytoplasm, which is most intensely coloured by the tetrazonium reactic The lipochondria could not be differentiated from the cytoplasm in any these tests.

Extracted neurones

Differential extraction methods were used to identify further the substanc in the lipochondria responsible for the reactions described above. Althou the solubilities of lipides in mixtures may be appreciably different from thc

---

FIG. 1 (plate). All photomicrographs are at the same magnification.

A, a motor neurone from the adult locust, showing the positive acid haematein reaction giv by the lipochondria.

B, acid haematein reaction on a section of ganglion extracted with hot acetone. All the ph pholipides have been displaced from the lipochondria and cytoplasm and have come to lie one region of the cell. A similar appearance is obtained after extraction with cold acetone.

C, acid haematein reaction on ganglia fixed in weak Bouin's fluid and extracted with py dine. All phospholipides have been extracted.

D, a neurone showing a positive PAS-reaction in the lipochondria. A similar result obtained after the PFAS test.

E, PAS reaction on ganglion extracted with cold acetone. Some PAS-positive lipide remai in the lipochondria. Some is displaced.

F, PAS reaction on ganglion extracted with hot acetone. All the cerebroside has be removed.

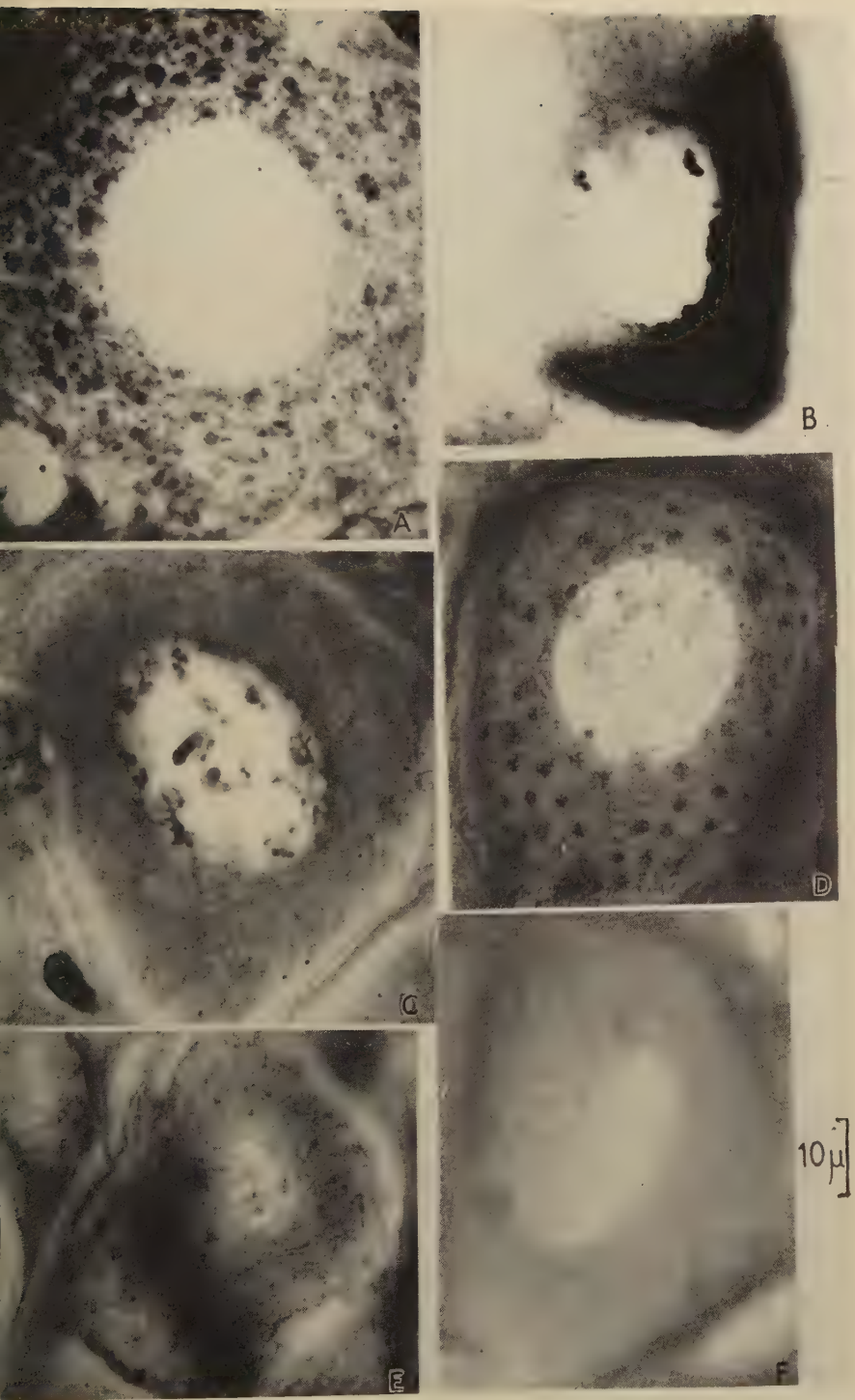


FIG. 1

S. A. SHAFIQ *and* W. G. B. CASSELMAN

f the components when in the pure state, extraction methods proved helpful n this case. They were applied to unfixed tissues because even fixation alters he extractability of the lipides. For example, when ganglia are fixed in ormaldehyde-calcium and then extracted with cold acetone, no lipide dis-lacement such as described below occurs.

After cold acetone extraction, Sudan black B shows that while some lipide emains in some of the lipochondria, a great amount has been displaced within he cells by the inward diffusion of the solvent. The lipide remaining in the ipochondria is PAS- (fig. 1, E) and PFAS-positive and acid haematein negative. The displaced lipide is strongly acid haematein positive and also weakly PAS- fig. 1, E) and PFAS-positive. The latter reactions are appropriately blocked y acetylation and bromination respectively.

After hot acetone extraction no lipide is demonstrable in the lipochondria y Sudan black B but the displaced lipide is still present. The acid haematein est also shows only the displaced lipide (fig. 1, B). The PAS (fig. 1, F) and FAS tests show nothing in the cells.

The ganglia were so distorted after extraction with hot ether that morpho-ogical details could not be distinguished.

In ganglia fixed in weak Bouin's fluid and extracted with pyridine, the lipo-hondria are not demonstrable by any of the above techniques. The cytoplasm s negative to the Sudan black B, PAS, and PFAS tests, but with the acid hae-natein test it is coloured diffusely dark brown and there are patches of similar olour in the nuclei (fig. 1, C), probably attributable to nucleic acids.

Only diffuse positive protein reactions are obtained in ganglia extracted with yridine. After cold and hot acetone extraction, however, the coupled tetra-onium reaction gives a stronger colour at the site of the displaced lipides han in the rest of the cell. It may be that some of the phospholipides are asso-iated with protein. There is not a demonstrable concentration of the latter n the lipochondria, which thus differ from the 'Golgi bodies' in the oocytes of he water-spider investigated by Krishna (1953).

From these observations it is concluded that the lipochondria contain two rincipal lipide components. The first is Sudanophil, acid haematein positive, AS- and PFAS-negative, and insoluble in cold or hot acetone, but soluble in yridine. This is identified as phospholipide. The second component is Sudano-hil, acid haematein negative, PAS- and PFAS-positive, insoluble in cold cetone but soluble in hot acetone and in pyridine. This is identified as un-aturated cerebroside. The faint PAS- and PFAS-reactions noted for the hospholipides displaced by cold acetone are attributed to traces of this cere-roside being dissolved in them.

Gersh (1948) and Arzac and Flores (1952) have considered that the positive AS-reaction given by the 'Golgi apparatus' in certain other cells is due to a arbohydrate moiety unassociated with lipides because it resists extraction vith fat solvents. Such a carbohydrate is not present in the lipochondria of the ocust motor neurones, where this reaction is entirely attributable to un-aturated cerebrosides. Lipide-pigments may also be PAS- and PFAS-positive

during their formation but they appear only in older locusts than were studie here, and they differ from the lipochondria in other histochemical propertie

The authors are grateful to Dr. J. R. Baker for suggesting this problem an for much helpful advice and discussion during the course of the work. It also a pleasure to thank Professor A. C. Hardy for accommodating us in th Department of Zoology and Comparative Anatomy at Oxford. This investiga tion was carried out while one of us (S. A. S.) was on a Rhodes Scholarshi and study leave from Dacca University, Pakistan, and the other (W. G. B. C was a Merck Postdoctoral Research Fellow in the Natural Sciences of th National Research Council of Canada, on leave from the University o Toronto, Ontario, Canada.

REFERENCES

Arzac, J. P., and Flores, L. G., 1952. Stain Tech., **27,** 9.
Baker, J. R., 1944. Quart. J. micr. Sci., **85,** 1.
—— 1946. Ibid., **87,** 441.
—— 1947*a*. Ibid., **88,** 115.
—— 1947*b*. Ibid., 463.
—— 1949. Ibid., **90,** 293.
Bensley, R. R., and Gersh, I., 1933. Anat. Rec., **57,** 217.
Brachet, J., 1953. Quart. J. micr. Sci., **94,** 1.
Cain, A. J., 1947*a*. Ibid., **88,** 383.
—— 1947*b*. Ibid., 467.
—— 1948. Ibid., **89,** 429.
—— 1949. Ibid., **90,** 411.
Casselman, W. G. B., 1952. Ibid., **93,** 381.
—— 1954. Ibid., **95,** 321.
Casselman, W. G. B., Macrae, A. I., and Simmons, E. S., 1954. J. Path. and Bact. (in th press).
Chèvremont, M., and Frederic, J., 1943. Arch. Biol., **54,** 589.
Chiffelle, T. L., and Putt, F. A., 1951. Stain Tech., **26,** 51.
Coujard, R., 1943. Bull. Hist. Appl., **20,** 161.
Danielli, J. F., 1947. Symp. Soc. exp. Biol., **1,** 101.
—— 1949. Cold Spr. Harb. Symp. quant. Biol., **14,** 32.
—— 1953. *Cytochemistry: a critical approach.* New York (Wiley).
Dempsey, E. W., Singer, M., and Wislocki, G. B., 1950. Stain Tech., **25,** 73.
Feulgen, R., and Rossenbeck, H., 1924. Zeit. Physiol. Chem., **135,** 203.
Gersh, I., 1948. Arch. Path., **47,** 99.
Hayes, E. R., 1949. Stain Tech., **24,** 19.
Jordan, B. M., and Baker, J. R., unpublished.
Keilig, I., 1944. Virchows Arch. Path. Anat., **312,** 405.
Krishna, D., 1953. Quart. J. micr. Sci., **94,** 315.
Lillie, R. D., 1944. Stain Tech., **19,** 55.
—— 1952. Ibid., **27,** 37.
Lillie, R. D., and Burtner, H. J., 1953. J. Histochem. and Cytochem., **1,** 8.
McManus, J. F. A., 1946*a*. Nature Lond., **158,** 202.
—— 1946*b*. J. path. Bact., **58,** 93.
Pearse, A. G. E., 1953. *Histochemistry: theoretical and applied.* London (J. A. Churchill).
Romieu, M., 1925. C.R. Soc. Biol., **92,** 787.
Serra, J. A., 1946. Stain Tech., **21,** 5.
Shafiq, S. A., 1953. Quart. J. micr. Sci., **94,** 319.
—— 1954. Ibid., **95,** 305.
Schneider, W. C., 1945. J. Biol. Chem., **161,** 293.
Tomasi, J. A. de, 1936. Stain Tech., **11,** 137.
Wolman, M., and Greco, J., 1952. Ibid., **27,** 317.

cetylated Sudan Black B as a More Specific Histochemical Reagent for Lipides

By W. G. BRUCE CASSELMAN

From the Cytological Laboratory, Department of Zoology and Comparative Anatomy, University Museum, Oxford)

Summary

Sudan black B is treated with an equivalent of acetic anhydride. The acetylated roduct is more specific for lipides than the parent dye.

ACETYLATED Sudan black B appears promising as a more specific reagent for the histochemical demonstration of lipides. This and other erivatives of Sudan black B, Sudan IV, and oil red O were introduced by illie and Burtner in 1953. Although the formulae for Sudan black B given y these authors and by Conn (1953) differ, they do agree on the presence of vo secondary amino-groups. These are, undoubtedly, the sites of acetylation.

Lillie and Burtner (1953) treated Sudan black B with a large excess of acetic nhydride in pyridine. With some brands of the dye, appreciable decom- osition occurs, yielding orange or brown substances which are not lipide olorants. This seldom occurs, however, if Kaufmann's (1909) method of cetylation is used with an equivalent of acetic anhydride in an inert solvent. he addition of sulphuric acid as a catalyst (Fieser and Martin, 1935) is nnecessary and, sometimes, appears to have resulted in an inferior product. he following method is suggested:

Dissolve 1 gm. of Sudan black B in 100 ml. of diethyl ether. Filter the olution to remove a lipide-insoluble fraction. Under a reflux condenser, heat ie etherial solution to boiling and add 0·5 ml. of acetic anhydride in 20 ml. of ther. Reflux for 20 minutes. Cool and filter the mixture. Transfer the filtrate o a separating funnel. Extract it repeatedly with cold water until the aqueous ayer is no longer coloured and is not appreciably acidic ('universal' indicator aper). Pour the solution of acetylated Sudan black B into a dish and evaporate ff the ether.

The black product has a metallic lustre. Like Sudan black B, it is stable in ropylene glycol and moderately stable in 70 per cent. ethanol. In paper hromatograms, its principal fraction is dark blue-black. There are small mounts of bluish-grey components and, occasionally, a trace of a brownish ne.

Acetylated Sudan black B may be used in the procedures described by aker (1944), Lillie (1944), or Chiffelle and Putt (1951). Prepared as described bove, it has been applied to a variety of histochemical analyses (Casselman, 954*a*, unpublished; Shafiq and Casselman, 1954). From all sites of colora- ion, the acetylated derivative has been readily extractable with 70 per cent.

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 321-322, September 1954.]

ethanol; whereas, with Sudan black B, 'stable sudanophilia' has been observe for some structures, especially the 'lipochondria' in locust motor neuron (Shafiq and Casselman, 1954). In these cytoplasmic inclusions at least, th retention of the dye appears to be associated with a high content of phosph lipides. Thus, the 'stable sudanophilia' first observed by Lillie and Burtn (1953) in the granules of leucocytes can, in some cases, be due to certain class of lipides. In other instances, however, it is probable that this is due to som non-lipide constituent with which the Sudan black B might react as a bas dye. Because of this possibility, it is suggested that acetylated Sudan black should be used in place of Sudan black B during histochemical analys of lipides, especially where 'stable sudanophilia' is demonstrable with th parent dye.

The greater specificity of acetylated Sudan black B for lipides has al proven valuable in certain histological studies and in paper chromatograp (Casselman, 1954*b*). In the case of the former, lipide-containing structur are still intensely coloured but there is little or no non-specific 'backgroun staining. This observation has been confirmed by Drs. C. L. Foster an R. R. Wilson who have kindly tested some of the preparations of acetylat Sudan black B.

The inspiration and guidance of Dr. J. R. Baker are gratefully acknov ledged.

These studies have been conducted during tenure of a Merck Postdocto Research Fellowship in the Natural Sciences of the National Research Coun of Canada.

The author's permanent address is: The Banting and Best Department Medical Research, University of Toronto, Ontario, Canada.

REFERENCES

Baker, J. R., 1944. Quart. J. micr. Sci., **85**, 1.
Casselman, W. G. B., 1954*a*. J. Roy. micr. Soc. (in press).
—— 1954*b*. Biochim. et Biophys. Acta, **14**, 450.
Chiffelle, T. L., and Putt, F. A., 1951. Stain Tech., **26**, 51.
Conn, H. J., 1953. *Biological stains*, 6th ed. Geneva, New York (Biotech. Publications).
Fieser, L. F., and Martin, M., 1935. J. Amer. Chem. Soc., **57**, 1838.
Kaufmann, K., 1909. Ber., **42**, 3481.
Lillie, R. D., 1944. Stain Tech., **19**, 55.
—— and Burtner, H. J., 1953. J. Histochem. and Cytochem., **1**, 8.
Shafiq, S. A., and Casselman, W. G. B., 1954. Quart. J. micr. Sci., **95**, 315.

Lead Tetra-Acetate/Schiff Tests in Histochemistry

By W. G. BRUCE CASSELMAN

From the Banting and Best Department of Medical Research, University of Toronto, Ontario, Canada, and the Cytological Laboratory, Department of Zoology and Comparative Anatomy, University Museum, Oxford)

SUMMARY

Certain polysaccharides, such as starch and glycogen, do not give consistently positive or negative reactions with all lead tetra-acetate/Schiff techniques. This depends upon the conditions under which the oxidant is used. A simple glacial acetic acid solution of lead tetra-acetate is least active but most specific. Added potassium acetate acts as a catalyst. Dilution with water not only increases the activity of the reagent but also decreases the specificity of the test.

VARIOUS histochemical techniques have been described in which lead tetra-acetate is used as a selective oxidant and followed by Schiff's reagent in a manner similar to the periodic acid/Schiff tests. Not all these lead tetra-acetate methods give the same results with certain polysaccharides. Thus, a positive reaction with glycogen has been reported to be obtained regularly with one technique (Shimizu and Kumamoto, 1952), occasionally with another (Lhotka, 1952, 1954), but not at all at room temperature with yet another (Glegg, Clermont, and Leblond, 1952). Unrecognized, such differences can be misleading in histochemical analyses. They are related to the composition of lead tetra-acetate reagent and the conditions under which it is used.

Comparative studies have been carried out with the methods of Crippa (1951), Shimizu and Kumamoto (1952), Jordan and McManus (1952), Lhotka (1952), Glegg, Clermont, and Leblond (1952), Hashim and Acra (1953), and Graumann (1953), as well as with four unpublished procedures. All of these techniques were tested on 10μ paraffin sections of potatoes and of mouse, rat, and rabbit livers and jejunums fixed in various reagents (formaldehyde-saline, absolute ethanol, Bouin's, Carnoy's, Champy's, Flemming's, Gendre's, Helly's, Rossman's, or Zenker's fluid). Those devised by Hashim and Acra (1953) were studied at 28° C., the temperature of these investigators' laboratory (Hashim, Acra, Afifi, and Shanklin, 1953). The effect of first covering the sections with collodion (Lison, 1953) was determined for all methods. Freshly prepared (Bailar, 1939) lead tetra-acetate was compared with three commercial products (Arapahoe Chemicals Co., Boulder, Colo., U.S.A.; Hopkins and Williams, Chadwell Heath, Essex; Light and Co., Colnbrook, Bucks).

With none of the procedures were there any appreciable differences between the results for the livers or jejunums from mice, rats, or rabbits or

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 323-325, September 1954.]

between those for potatoes purchased in Canada or England. The preservatic of the various polysaccharides, especially glycogen, varied to some degre with the fixative but their reactivities were unaffected by this or the collodic film. The latter favoured the retention of Schiff-positive oxidation product but with some methods it also gave a positive reaction. Although differing i assay (Dimroth and Schweitzer), the four samples of lead tetra-acetate ga similar histochemical results.

The differences between the observations for certain polysaccharides in th tissues could be related to the conditions under which the lead tetra-aceta is used in each method. Where the reaction medium is glacial acetic acid as i the procedures of Crippa (1951), Glegg, Clermont, and Leblond (1952), an Graumann (1953), or glacial acetic acid diluted with toluene or benzen as suggested by Hashim and Acra (1953), starch and glycogen do not reac positively under the specified conditions for each procedure. When anhydrou potassium acetate is added (Lhotka, 1952), these polysaccharides occasionall give a weakly positive reaction, especially when the duration of oxidation i prolonged. More strongly positive reactions are usually noted with th methods of Hashim and Acra (1953) in which the stock acetic acid solution c lead tetra-acetate is diluted with water. Intensely positive reactions are alway obtained when the diluent is aqueous sodium acetate as used by Shimiz and Kumamoto (1952) and by Jordan and McManus (1952). With a methods, hepatic and intestinal connective tissues, intestinal mucin, an potato cellulose react positively.

These observations demonstrate that while acetate can serve merely as catalyst for oxidation by lead tetra-acetate, water not only accelerates th reaction but decreases the specificity of the method (compare Fuson, 1950) This has also been noted by Lhotka (1954). The interpretation of histo chemical tests is usually more certain when these are carried out unde conditions giving results which conform with well established organic o biochemical observations. For example, starch is usually considered to b oxidized only with difficulty by lead tetra-acetate although readily by periodat (Criegee, 1948). The lead tetra-acetate/Schiff methods introduced by Cripp (1951), Lhotka (1952), Glegg, Clermont, and Leblond (1952), and Graumann (1953), and the methods of Hashim and Acra (1953) in which the diluent i benzene or toluene, may be preferable for critical applications. With th techniques of Shimizu and Kumamoto (1952), Jordan and McManus (1952) and those of Hashim and Acra (1953) where water is the diluent, carefu consideration should be given to the increased activity of the reagent and th decreased specificity of the methods. In all cases, any report of observation with a lead tetra-acetate/Schiff test should include details of the exact metho employed.

Additional studies have shown that not only the reaction medium but als the concentration of the lead tetra-acetate and the duration and temperatur at which it is used may affect the results. Careful adjustment of these variables however, provides a series of tests valuable for the comparative study o

carbohydrates and other tissue constituents containing appropriate reactive groups.

It is a pleasure to acknowledge the advice of Dr. J. R. Baker and Professor E. Baer, and the technical assistance of Miss B. M. Jordan and Mr. O. F. George.

These studies were conducted during tenure of a Senior Medical Research Fellowship (1952–3) and a Merck Postdoctoral Research Fellowship in the Natural Sciences (1953–4) of the National Research Council of Canada.

The author's permanent address is the Banting and Best Department of Medical Research, University of Toronto, Ontario, Canada.

REFERENCES

Bailar, J. C., 1939. *Inorganic syntheses* (ed. by H. S. Booth), **1**, 47. New York (Wiley).
Criegee, R., 1948. *Newer methods of preparative organic chemistry*, p. 1. New York (Interscience).
Crippa, A., 1951. Boll. Soc. Ital. biol. sper., **27**, 599.
Glegg, R. E., Clermont, Y., and Leblond, C. P., 1952. Stain Tech., **27**, 277.
Graumann, W., 1953. Mikroskopie, **8**, 218.
Hashim, S. A., and Acra, A. N., 1953. Ibid., **28**, 1.
——, ——, Afifi, A. K., and Shanklin, W. M., 1953. Ibid., **28**, 113.
Jordan, R. H., and McManus, J. F. A., 1952. J. Nat. Cancer Inst., **13**, 228.
Lhotka, J. F., 1952. Stain Tech., **27**, 213.
—— 1954. Personal communication.
Lison, L., 1953. *Histochimie et cytochimie animales*, 2nd ed. Paris (Gauthier-Villars).
Shimizu, N., and Kumamoto, T., 1952. Stain Tech., **27**, 97.

Metachromatic Dye-Substrate Interactions

By BENGT SYLVÉN

(*From the Cancer Research Division of Radiumhemmet, Stockholm* 60, *Sweden*)

Summary

From the interactions between azure A and various substrates with different physical d chemical characteristics, some suggestions are made as to the nature of the etachromatic colour reaction. The following essential factors are necessary for the pearance of metachromasia.

1. The *orderly* alignment of dye molecules is favoured if the molecules present drophobic and hydrophilic parts.

2. The distribution of electro-negative surface charges of the substrate determines the ignment of dye molecules and the degree of metachromasia. A certain minimum inter-harge distance (interplanar), possibly about 5 Å, is a prerequisite for metachromasia. he degree of metachromasia increases when the intermolecular distance between djacent dye molecules aggregated to the substrate surface becomes less than 5 Å.

3. The presence of water is essential for metachromatic interaction.

The observed spectral absorption shifts are explained by the assumption that new onds appear between adjacent dye molecules. The necessary energy requirements are f the order of about 8 cal./mol. In the case of azure A, various types of intermolecular onds are discussed: hydrogen bonds or perhaps hydrogen bonds plus oxygen-sulphur ridges. It seems very likely that water molecules may be intercalated between adjacent ye molecules as suggested by Scheibe and Sheppard.

Since the dye and many organic substrates form polydisperse colloidal solutions and nce further the dye-substrate interactions often imply the appearance of micellar ggregates and precipitates in *in vitro* experiments, *spectrophotometric data should be terpreted with great caution*. Evidence is presented indicating that *the heterogeneity of e sol systems in many instances renders spectrophotometry useless*. The desired colour anges are instead conveniently registered by microscopy with transmitted light.

Available data indicate that the electro-negative surface charge density is the ssential characteristic of the various substrates for the conditioning of metachromatic ye-substrate interaction.

Contents

I. Introduction

METACHROMATIC colour reactions of basic aniline dye-stuffs with particulate substrates involve two principal steps: first, interaction between dye and substrate molecules; and secondly, interaction between

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 327-358, September 1954.]

adjacent dye molecules aggregated to the substrate. The real nature of the colour shift phenomena is poorly understood, and cannot be investigated by direct measurement by means of methods at present available. The present approach is to study these interactions over a wider range of organic and inorganic substrate compounds by indirect methods. Thus, it would seem fruitful to investigate the physical and chemical characteristics of various substrates giving rise to metachromatic colour reactions. This contribution should be regarded as a progress report preliminary to further, more exact, research. Present observations support a hypothesis on the nature of the metachromatic colour reaction quite similar to that previously presented by Sheppard (1942). For a detailed review of previous theories and experiments the readers are referred to papers by Hansen (1908), Schwarz and Herrmann (1922), Michaelis (1926), Holmes (1928), Lison (1935), Scheibe (1938), Bank and Bungenberg de Jong (1939), Spek (1940), a series of papers by Michaelis and co-workers during 1943–50, and others. Pertinent histochemical implications will be discussed elsewhere.

In this paper *metachromasia* will be used to indicate colour shifts from the orthochromatic level to longer wavelength regions as seen by *transmitted* light only. Colour shifts to shorter wavelengths, so-called 'negative metachromasia' (Lison and Mutsaars, 1950), will not be considered. Colour shifts from the orthochromatic level (blue) to longer wavelengths (red) are normally measured in terms of the absorption spectrum of the system, in which case the metachromatic shift is from longer to shorter wavelengths. All colour variations due to mixtures of dye-stuffs, coloured substrates, and emission of light (fluorescence), are omitted from this subject.

Adsorption is used in a wide sense including all reversible interactions between dyes and colloidal (micellar) surfaces. Since the actual type of linkage involved cannot be stated, there can be no clear distinction between adsorption, thus defined, and the common term 'complex formation'.

The tables referred to in the text are printed at the end of the paper.

II. Outlines of the Problem

Cationic dye-stuffs like azure A (fig. 1) and pinacyanol (fig. 2) form colloidal aqueous solutions presenting degrees of molecular aggregation depending on the concentration and environmental conditions (Ostwald, 1933; Robinson, 1935; Valkó, 1935; Scheibe, 1938; Rabinowitch and Epstein, 1941; and others). The dye molecules have a large hydrophobic (organophilic) and a small hydrophilic part, which condition favours the orderly alignment of molecules. Aggregation is further favoured by high dye concentration, and alkaline pH (increasing hydration); on the other hand, aggregation is hindered by increase in ionic strength either from the action of a 'substrate' or other ions, by the addition of salts and acids, and by the supply of thermal energy. In the case of aggregation associated with metachromasia, the spectral absorption shift to shorter wavelength regions probably indicates that new bond

th higher bonding energy have appeared between the aggregated dye mole-
les. The reversibility and low energy of these bonds suggest that in most
ses they are of long-range type (van der Waals forces). The exact types of
nds are, however, not known, and measurements of intermolecular distances
ve not been performed. Furthermore, it is possible that the type of bonds

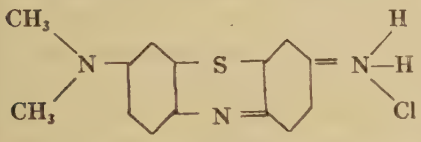


FIG. 1.

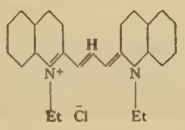


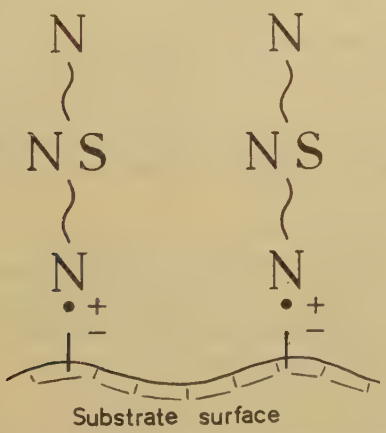
FIG. 2.

FIG. 1. Formula of azure A (dimethylthionine) = NH_2Cl represents the hydrophilic part of the molecule; the rest is hydrophobic.

FIG. 2. Formula of the quinoline dye pinacyanol ('sensitol red').

sponsible for metachromasia varies under different conditions and for differ-
nt dyes, thus depending in part on the molecular structure of the dye in
uestion. The spectral characteristics of pure aqueous dye solutions are con-
eniently defined by absorption spectrophotometry.

When, however, the discussion is extended to include dye-substrate inter-



IG. 3. Schematic presentation of the proposed monolayer alignment of azure A molecules ttached to the surface of a substrate with electro-negative charges available. The expected intermolecular bonds are discussed in the text.

ctions, the characteristics and purity of the particular substrate will influence he results. In the case of hydrophobic substrates no metachromatic reaction vill occur. In the case of colloidal substrates in general, adsorption phenomena ake place (see Merrill and Spencer, 1948; and others). Some inorganic sub-trates lead to the formation of more stable metachromatic 'complexes' of the ype mentioned in Section VI.

Clearly, in the case of biocolloidal substrates metachromasia as well as more abile adsorption phenomena may involve very similar colour changes. Thus, in order to distinguish between these effects, it is necessary to introduce arbitrary

qualifications referring to the *stability* of the newly-formed metachromatic d aggregates in a sense previously expressed by Lison (1935) and others. Th procedure has been unduly criticized by various authors, but it should recognized that the stability of metachromatic aggregation refers to the inte molecular bonding energies. In the case of weak adsorption of dye molecul associated with some metachromasia the colour reaction will be easily i hibited or reversed by addition of salt or ethanol under conditions when mo stable metachromatic phenomena still prevail. It appears that such simp 'stability tests' will give useful information not easily obtained by other mea

In this report on metachromatic dye-substrate interactions the followi main factors will be emphasized:

The dye-stuff, its chemical and physical characteristics, and its tendency to mol cular aggregation (or complex formation);
The particular substrate, its chemical and physical properties, and its molecul configuration in the sol and gel states;
The presence of water;
Environmental factors, such as pH, ionic strength, temperature, &c.
The stability of the spectral shifts, viz. of the new dyestuff aggregates.

In order to explain the fundamental role of water in metachromatic d aggregation Scheibe and Sheppard proposed that one water molecule might l intercalated between adjacent dye molecules (fig. 3), thus implying an inte planar distance of about 4 Å. From an energetic point of view and from t data on the stability of certain dye aggregates reported below, it is possible th other means of hydrogen bonding and also other types of linkages in so cases may be involved.

It is the scope of the present investigation to study metachromasia by usi mainly one well-known dye-stuff and a collection of substrates with differe characteristics in order to find some common denominator in those substra indicative of a governing principle. This aspect was touched upon in a previo report (Sylvén and Malmgren, 1952).

III. Materials and Methods

Various substrates with well-known composition and high degree of purit and reasonably salt-free, have been tested for metachromasia as diluted so concentrated gels, and/or solid films. Most of the organic substrates prese colloidal properties necessitating special attention to particle size. Experimen were performed in dilute aqueous solutions of azure A (dimethylthionin National Aniline Co. certified stain, lot NAz 13) and in a few cases also wi pinacyanol (Eastman Kodak Co.). Spectrophotometric data (Beckman Mod DU or Hilger Uvispek) refer mainly to dye-substrate reactions performed sol state. Absorption measurements on stained films are briefly reported. A absorption data are supplemented by microscopic observations on the colour precipitates appearing as a result of dye-substrate reactions.

In order to assess the degrees of stability of metachromatic dye-substrate ggregates, the labile adsorption phenomena are inhibited or reversed in a eparate series of experiments by either increase of temperature, addition of lt, or addition of 10–60 per cent. ethanol. However, by these procedures, oth the state of the dye and the substrates will unfortunately be affected.

A number of polysaccharides such as gum arabic and other 'mucilages' of ndefined composition have for obvious reasons been omitted. They were often sed as test objects of metachromasia by previous investigators (Bank and Jungenberg de Jong, 1939; Michaelis and Granick, 1945; Michaelis, 1950; nd others).

The experimental data are, for the sake of brevity, compressed and have een classified according to the physical and chemical characteristics of the ubstrates. Detailed spectrometric evidence will be presented only when mportant points have to be illustrated.

IV. Hydrophilic Organic Compounds

The data presented in tables 1–6 and figs. 4–8 indicate that substrate characeristics, viz. electro-negative surface charge density, particle size, and ther physical conditions will influence the mode of aggregation of dye molecules, and thus determine the resulting colour. Some substrate characteristics will be discussed below, although extensive physical data on colloidal charge lensity cannot be obtained.

Surface charge density. Metachromatic dye-substrate aggregates seem to occur only when the substrates have free electro-negative surface charges availble. Non-charged sugars and polysaccharides display metachromasia neither n the sol nor in the gel state (table 1); they form, however, in many cases orthochromatic dye-stuff 'complexes' of varying stability. Substitution of weak negative radicles such as $—CH_2.COO^{1-}$ and $—COO^{1-}$ induces metachromatic reactions at first only in the gel state (tables 2–4). This is illustrated in the case of hyaluronic acid (see table 3 and previous detailed data by Sylvén and Malmgren, 1952). The cellulose fibre studies (table 4) illustrate how the degree of metachromasia increases with increasing degrees of carboxymethylation. Studies with polarized light indicate that metachromasia appears in those fibre foci where substitution takes place (see Frey-Wyssling, 1936). Metachromatic transverse 'chromosome-like' bands are often observed associated with loss of birefringence. By the introduction of a sufficient number of carboxyl, phosphate, or sulphate radicles a condition is reached when such substrates show metachromasia also in the sol state (tables 2, 4, and 5; figs. 4–8). A fairly close correlation is found between the degrees of substitution (atoms per period of polysaccharide) and metachromasia (table 9). This implies that a minimum intercharge distance is necessary for the appearance of metachromasia. This would explain why gelation and micelle formation may be accompanied by metachromasia in the case of low-charged colloids.

Charge quality. The degree of metachromasia seems also to depend on the

quality of the substituted charged groups. Comparing polysaccharides similar unit dimensions with one substituted group per period, e.g.

$$R—CH_2.COO^{1-} < R—COO^{1-} < R—OPO_3^{2-} < R—OSO_3^{2-},$$

a series of increasing degrees of metachromasia may be obtained.

The available valences do not explain why ester sulphates on the whole di play more stable and pronounced metachromasia than other radicles. Thu

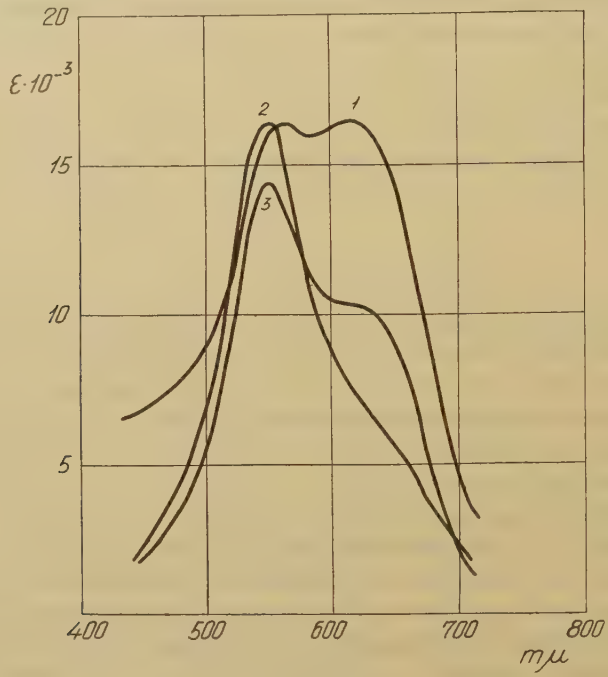


FIG. 4. Spectral shift of azure A and pectinate.

Conditions: Na-salt of pectinate, 2×10^{-5} mol azure A in aqueous solution, temp. 20° C. Mixed red and blue precipitates appear in all tubes. Maximum absorption at 545 mμ. Amounts of substrate: No. 1, 5 mg./ml.; No. 2, 0·25 mg./ml.; No. 3, 0·05 mg./ml.

for example, one sulphate group per dextran period gives a degree of met chromasia similar to that given by two phosphate groups per period.

Quantitative dye-substrate ratio. Previous and present data indicate that th ratio is another important factor for the appearance of metachromatic re actions. A certain threshold amount of dye is apparently necessary befo metachromatic colour shifts and metachromatic dye-substrate aggregate occur (cf. fig. 6, A and B). Sub-threshold amounts of dye will in the case heparin, for example, produce orthochromatic aggregates. Further, there microscopic evidence of the fact that excess of dye may be adsorbed to th surface of an already metachromatic substrate nucleus. This excess may sho purely orthochromatic colour, and is easily removed by adsorption repellant The minimum number of dye molecules necessarily aggregated to eac

arged focus in order to produce metachromasia is not known (see Lison and utsaars, 1950), and hence the real significance of dye-substrate ratios can-t be discussed. Quantitative data on the relation between degrees of meta-

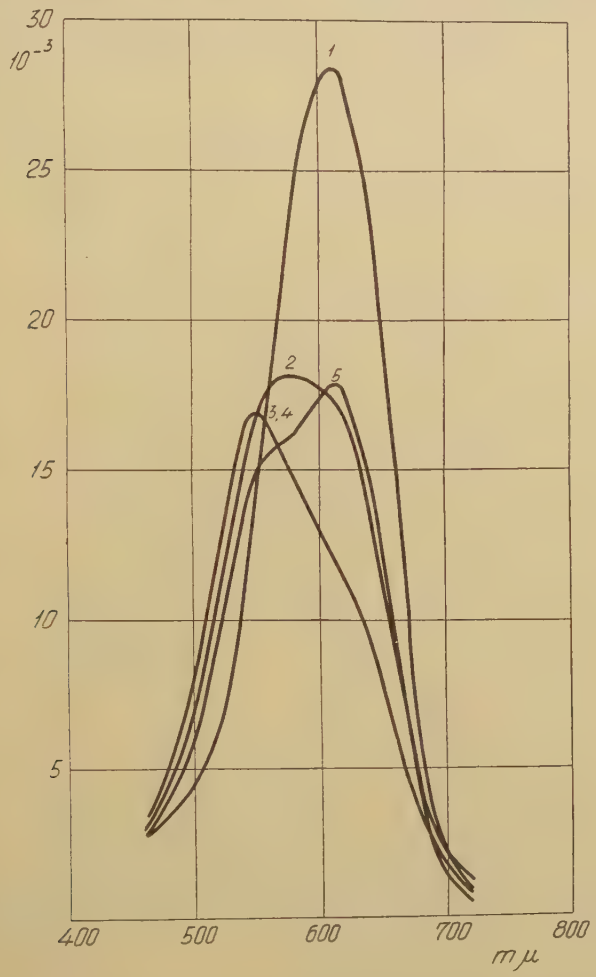


FIG. 5. Metachromasia of chondroitin sulphate to azure A.
Conditions: varying amounts of chondroitin sulphate, highly purified (analytical data stated by Sylvén and Malmgren, 1952). 2×10^{-5} mol azure A in aqueous solutions, temp. 20° C. Mixed red and blue precipitates appearing with substrate.
No. 1, No substrate; No. 2, 1 mg./ml.; No. 3, 0·25 mg./ml.; No. 4, 0·05 mg./ml.; No. 5, 0·005 mg./ml.

hromasia and mono- or multilayer aggregation of dye is thus required. Equi-olar ratios, as discussed by Levine and Schubert (1952), are not expected to e valid in cases when the substrates bear several negative charges per period.

Colour of aggregates. Sols and gels of noncharged and some low-charged olysaccharides (tables 1 and 2) will on the addition of dye produce ortho-

chromatic dye-substrate precipitates, which often appear quite slowly. Un
similar conditions chromotrope substrates form aggregates presenting m
colours as seen in transmitted light under the microscope (tables 2–5), but
whole of the substrate surface is not necessarily metachromatic; it may be se

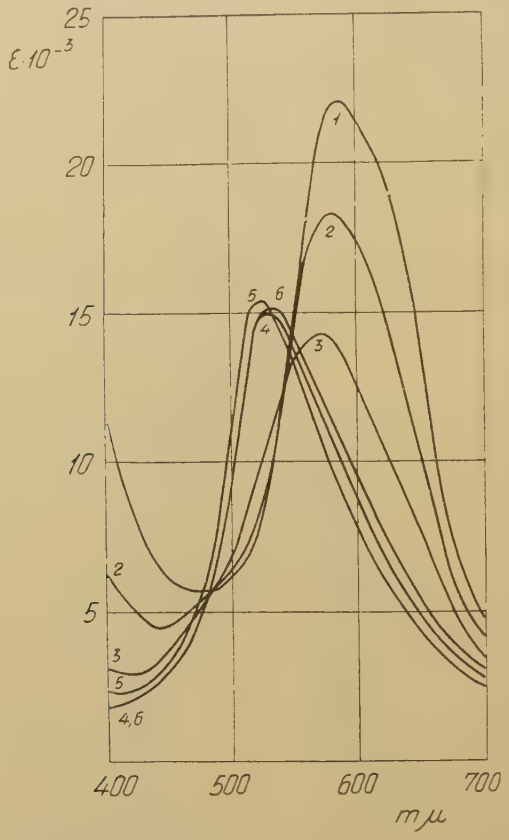
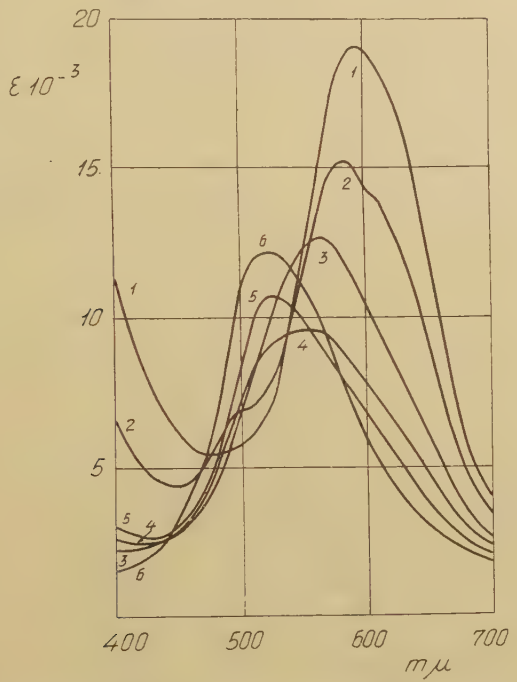


Fig. 6, a. Interaction of heparin (Vitrum) and azure A.
Decreasing amounts of heparin added to aqueous salt-free 3×10^{-5} mol solutions of azure
Spectral shifts registered shortly afterwards. Heparin concentrations as follows:

Curve No.	1	10 mg./ml.	
,,	2	5	,,
,,	3	2	,,
,,	4	1	,,
,,	5	0·5	,,
,,	6	0·25	,, (5×10^{-4} g. mol)

that parts are stained blue and other parts red (see Section V). Strongly met
chromatic substrates (table 5) generally show a uniform metachromasia
over the surface of the precipitated aggregates, provided a 'sufficient' amou
of dye is added. This implies that we have a sliding scale of metachromat
substrates (sol state) forming partly blue and partly red coloured aggregat
within the range of spectral absorption (between 615 and 540 mμ, approx
mately).

Evaluation of absorption measurements. Since the colloidal properties of the bstrates are very different, and minute dye-substrate aggregates are formed, may be foreseen that some sols are so heterogeneous that spectral absorption ta alone cannot give a true picture of the relevant colour changes. Absorption d microscopy data on a series of various concentrations of polyelectrolytes ch as chondroitin sulphate (fig. 5 and table 6) and heparin (fig. 6) will



IG. 6, B. Absorption curves on supernatants obtained after centrifugation of same solutions r 60 minutes at 40,000 r.p.m. (SPINCO). Marked decrease in extinction values should be oted. Yellow colour of concentrated heparin solutions explains the absorption below 450 mμ.

No.	*Visible colour of solutions*	*Colour of sedimented precipitates*
1	Yellow+blue = green	Faint blue
2	Bluish-green	Mainly blue+traces of a red component
3	Blue with a shade of red	Blue+increasing amounts of a red component
4 and 5	Violet	Blue and red components
6	Violet	All precipitates intensely red-stained

lustrate these points. Comparison of extinction values and relative extinction atios between different absorption bands (Levine and Schubert, 1952) seem lso to be of doubtful significance owing to this heterogeneity of the sol ystems. The presence and effects of suspended dye-substrate microaggre-ates is further illustrated by the sedimentation experiments presented in g. 6, B. It may further be recalled that location of absorption maxima and xtinction values are easily influenced by environmental conditions (table 6 nd fig. 7), which are sometimes difficult to control.

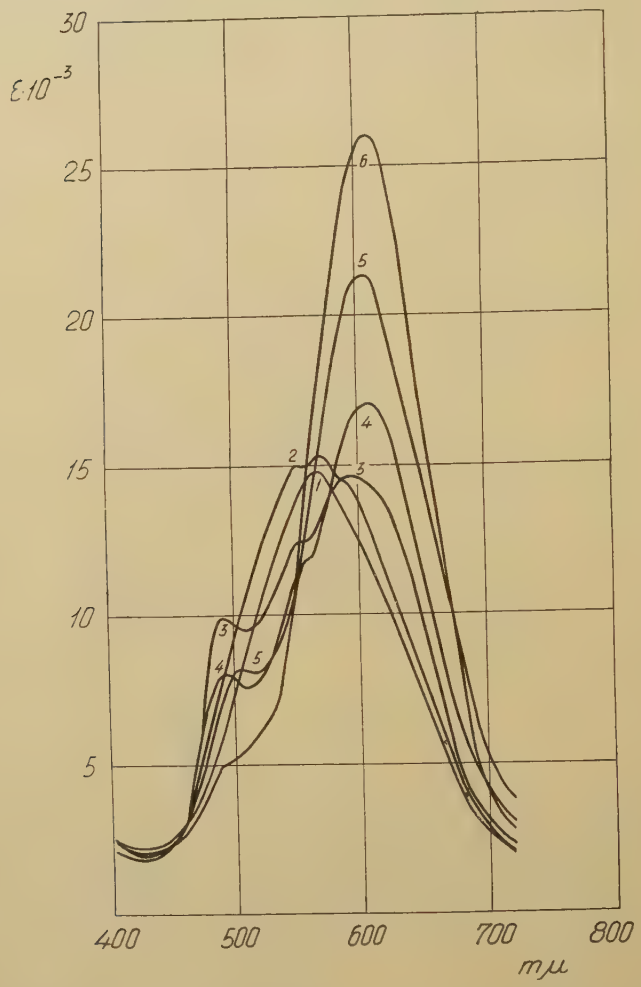


FIG. 7. Influence of ionic strength on the interaction between heparin and azure A.

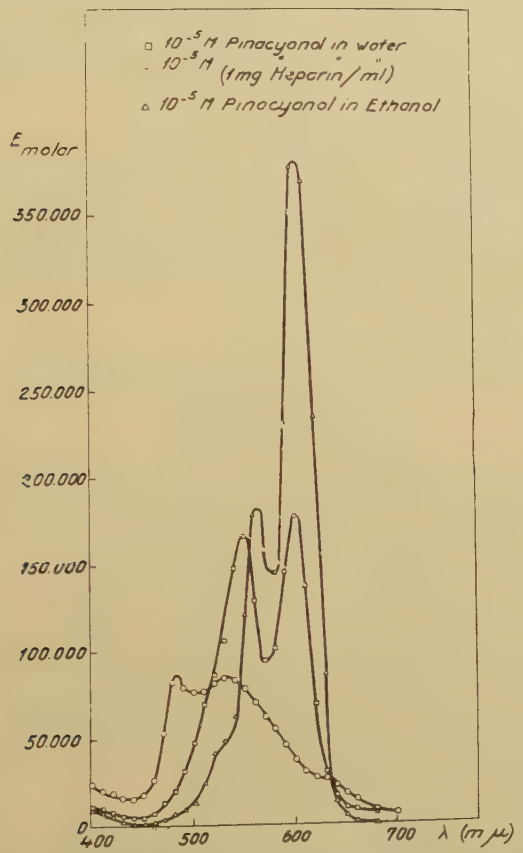
The conventional terms for the designation of absorption band maxima are only used convenience.

Standard conditions: heparin 2 mg./ml. 2×10^{-5} mol azure A in aqueous solution, te 20° C.

No.	*Medium*	λ_γ	$\epsilon_{\gamma\,max}$	*Visual colours*	*Colours of precipitates*
1	H_2O+heparin	Not defined	..	Violet	Red
2	0·003 mol NaCl	,,	..	Violet	Red
3	0·03 ,,	490	0·195	Violet	Red
4	0·09 ,,	490	0·160	Blue	Blue and red
5	0·18 ,,	500	0·146	Blue	Blue and small amount of
6	0·3 ,,	(500)	(0·1)	Blue	Only blue

Evaluation of absorption data on chromotrope sols would therefore be advisable er additional information has first *been obtained by means of microscopy.* New sorption peaks at shorter wavelengths (β- and γ-bands) do indicate the currence of new dye aggregates in the system after addition of the substrate.

Stability of metachromatic reactions. Stability is used to denote the force



IG. 8. Spectral shift of pinacyanol HCl in aqueous solution after addition of heparin is shown for comparison. In this case also, marked brick-red precipitates are obtained.

ecessary to prevent or to reverse metachromatic reactions (see Section II). ven markedly metachromatic substrates are extremely sensitive to increase ı ionic strength, and a smallish amount of salt is often sufficient to abolish or revent a metachromatic reaction. On the other hand, most metachromatic eactions of moderately charged compounds stand ethanol treatment at concentrations up to 30 per cent. approximately. Chondroitin sulphate (table 6) hows less stable metachromasia than most other ester sulphates. Some data n salt inhibition of chondroitin sulphate and heparin metachromasia are ound in table 6 and fig. 7. It should further be noted that the acid poly-

saccharides bind considerable amounts of salt, which is seldom complet removable.

The influence of salt has not been studied very carefully. The approxim stability against ethanol at varying concentrations is mentioned in tables 2 Thus it is likely that there is a sliding scale of different degrees of stabil The ester sulphate polysaccharides with the highest metachromatic ene levels (tables 5 and 9) show the most marked stability, and these metach matic dye-substrate aggregates just turn bluish in colour at ethanol concent tions above 50 to 60 per cent. (see table 5). It is typical that this effect reversed after replacement of water.

It is considered that the inhibitory effect of low salt concentrations depe mainly upon competition with the anionic charges of the substrates. On t other hand, ethanol seems mainly to act by withdrawal of water (possil necessary for the new intermolecular bonds between adjacent dye-stuff mo cules), thus temporarily destroying metachromasia, but not the dye-substr linkages.

V. Organic Compounds with Hydrophilic and Hydrophobic Characteristics

In this section, a heterogeneous group of other compounds, presenti different structural and colloidal properties, will be discussed. The behavic with water and cationic dyes is largely determined by the presence of hyd phili c and hydrophobic groups in the molecular skeleton. Data are present below as to the role of surface charge density and molecular orientati (micelle formation) influencing the metachromatic colour reaction.

Nucleotides and nucleic acids. Partially purified Ca- and Na-salts of A gave no metachromatic colour shifts, and the precipitates obtained are orth chromatically blue. The main components are ATP, ADP, and adenylic a (Burrows and others, 1952). The undefined metachromatic admixture (table seems to correspond to ortho- or pyrophosphate.

The results with RNA and DNA recorded in table 7 and figs. 9 and 10 a of special interest. The number of phosphate radicles and presumed statisti intercharge distance of about 3·4 Å would in both substrates seem sufficie to produce a marked degree of metachromasia, but this is apparently not t case. The spectral shift in absorption maximum to about 570 mμ remains u explained since this does not correspond to the orthochromatic colour of t precipitates which occur, nor to the colour of stained RNA gels or film Recent claims by Flax and Himes (1951) that tissue RNA (rather RNP) sho metachromasia to azure B (trimethylthionine) cannot be discussed for lack detailed data.

In the case of highly purified DNA the following results will be considere (*a*) In diluted solutions the observed decrease in extinction values (fig. 1 indicates that some dye-stuff has been adsorbed to the substrate. (*b*) The r sulting precipitates appear as non-orientated membraneous sheaths presenti

parts both red and blue colours. (*c*) Staining of non-oriented gels and films ows in parts strongly metachromatic and other orthochromatic areas. In as of low birefringence the metachromasia seems to be most marked.

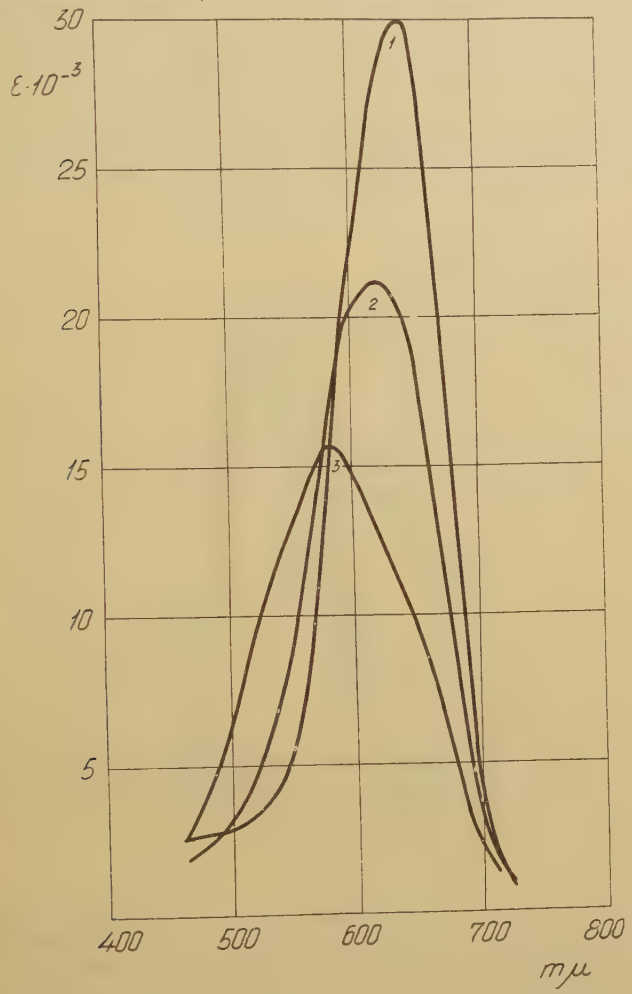


FIG. 9. Absorption spectra of azure A (3×10^{-5} M.) at varying concentrations of RNA.
No. 1 2·5 mg./ml. Absorption maximum at 640 mμ. Visual colour green. No precipitates found.
No. 2 0·125 mg./ml. Absorption maximum at 620 mμ. Visual colour and precipitates blue.
No. 3 0·025 mg./ml. Absorption maximum at 580 mμ. Visual colour blue with a shade of violet. Microscopic precipitates still blue.

d) Strongly birefringent DNA fibres produced by the 'cover-slip' method show uniform and very marked bright red metachromasia. Staining in aqueous media naturally implies swelling of fibres and loss of orientation. Detailed data on the possible reappearance of birefringence during subsequent drying

have not been secured. The observed metachromasia of purified DNA extremely salt-sensitive but less sensitive to addition of ethanol. The absor tion maximum of such metachromatic areas of DNA films and fibres

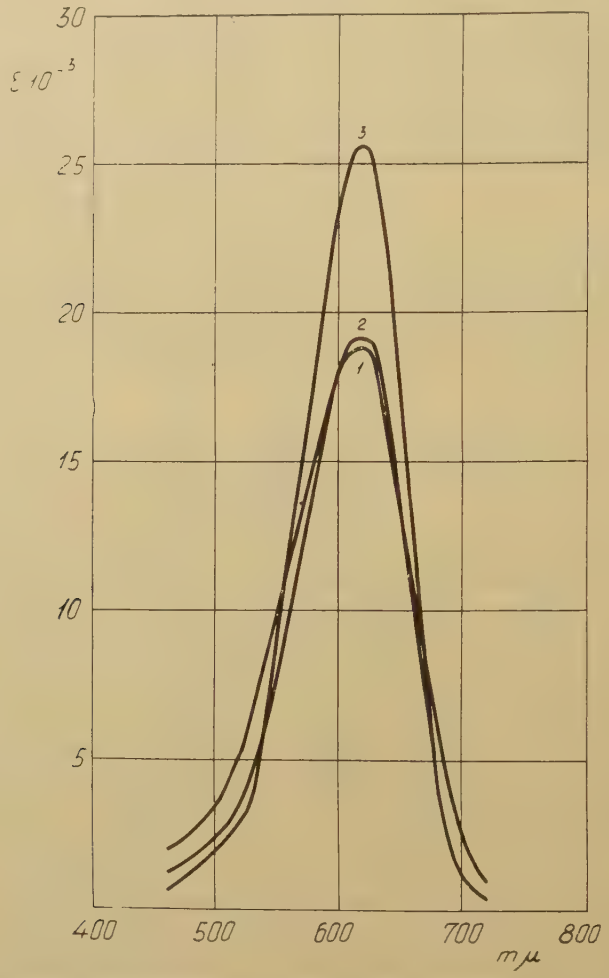


FIG. 10. No spectral change of azure A (3×10^{-5} M.) at varying concentrations of hi molecular DNA.

Same amounts of DNA as for RNA in fig. 9.
pH of solution about 6.
In No. 1 and 2 the microscopic precipitates are blue.
In No. 3 (excess of dye) the membranous precipitates are partly red but in places blue

estimated at about 540 mμ, a similar level to that given by chrondroit sulphate. (*e*) If a non-oriented (non-birefringent) multilayer film of DNA 'over-stained' in aqueous solution of azure A—a condition illustrating a hig 'dye-substrate ratio'—the previously mentioned bright red metachromas changes to a dark violet colour (see also Lison and Mutsaars, 1950), indicati

the red and blue components mentioned under (*c*). It is felt that the apparent crease of blue components would most likely be due to a multilayer aggregation of dye molecules (see fig. 3), to be discussed below.

According to Carnes, Weissman, and Rubin (1951), both DNA (partially urified) and DNP would give alcohol-resistant metachromasia with a γ-band aximum at 560 mμ '*in toluidine blue solutions at proper* pH, *ionic strength, &c.*'. his location of absorption maximum would indicate a weak degree of metaromasia comparable to that of carboxyl-bearing polysaccharides. It may be easonable to expect that their figure is obtained under conditions when DNA cks orientation (see above, points (*a*) to (*c*)).

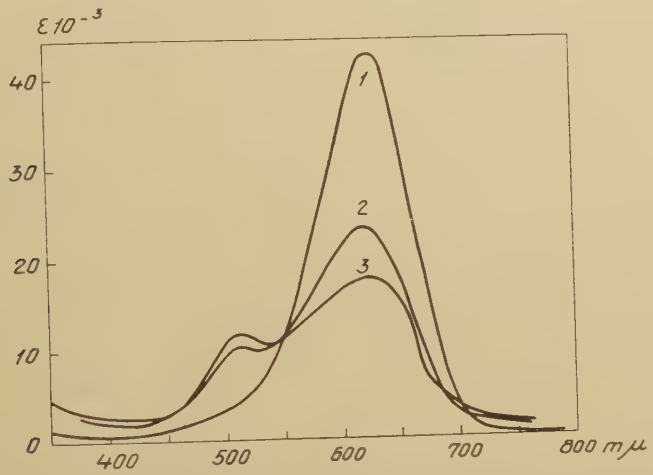
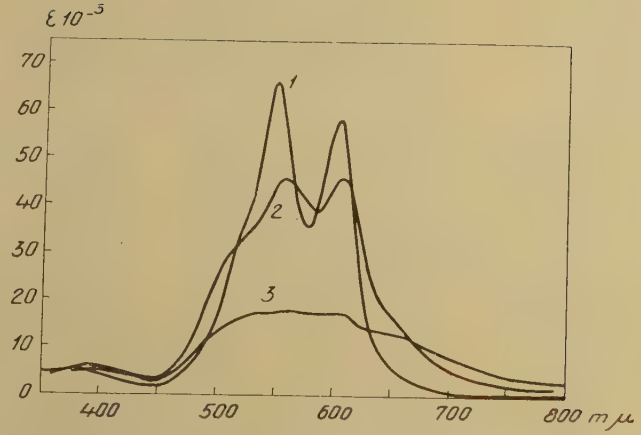


FIG. 11. Absorption measurements of azure A ($1{\cdot}54 \times 10^{-5}$ mol/lit.) in aqueous solutions with addition of Na-triisopropyl-benzenesulphonate. Hilger spectrophotometer, 5 mm. cuvettes.

No.	TPBS *mol per lit.* $\times 10^{-3}$	pH
1	0–0·9	5·8
2	9	5·7
3	35	6·4

These observations on nucleic acids indicate that *the state of molecular orientation of the particular substrate is of great importance for the metachromatic colour reaction.* It is further suggested that *the hydrophobic purine parts of the molecular skeleton interfere with dye aggregation to the charged sugar-phosphate groups*, thus explaining the orthochromatic characteristics of RNA and the dual behaviour of DNA in the gel state. More detailed physical studies are, however, needed to elucidate these questions. The model experiments reported above are not directly transferable to the various nucleic acid/protein complexes found in tissue material.

Metachromasia of some detergents. The metachromasia of soaps with hydrophilic groups attached to the ends of the hydrophobic molecules (sodium oleate and Aerosol) was extensively studied by Michaelis. Under suitable quantitative conditions spectral shifts, denoted as γ-bands, were observed at



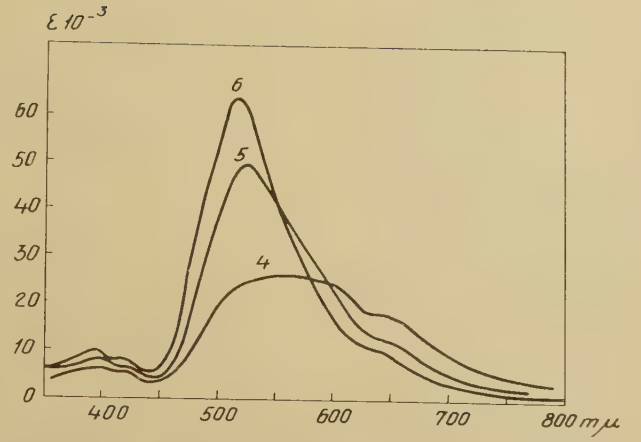


FIG. 12. Absorption measurements of pinacyanol HCl ($1{\cdot}99 \times 10^{-5}$ mol/lit.) in aqueous solutions with addition of Na-triisopropyl-benzene-sulphonate. Hilger spectrophotometer, 5 mm. cuvettes.

No.	TPBS *mol per lit.* $\times 10^{-3}$	pH
1	0	6·3
2	0·09	5·2
3	0·9	5·2
4	2·2	5·5
5	8·8	5·7
6	35	6·4

bout 580 mμ with toluidine blue, and this was considered as evidence of nicellar staining'. The role of weak adsorption phenomena and the instability f these colour shifts towards salts and ethanol was, however, not emphasized. ery marked spectral shifts have also been studied in aqueous solutions of inacyanol chloride and various anionic soaps in order to determine the so-alled 'critical concentration for the formation of micelles' (Corrin, Klevens, nd Harkins, 1946; Corrin and Harkins, 1947; Shuck and Lingafelter, 1949; nd Herzfeld, Corrin, and Harkins, 1950). These physical data, and those of IcBain (1940) and of others tend, however, to indicate that micelle formation n the part of various soaps occurs over a wide concentration range.

In figs. 11 and 12 the results of experiments are recorded on the spectral ehaviour of azure A and pinacyanol chloride in aqueous solutions of a micelle-orming detergent TPBS (Na-triisopropyl-benzene-sulphonate and isomers, btained by courtesy of Dr. Gordon Shuck, Montana State University, J.S.A.). It is seen that marked spectral shifts to shorter wavelengths occur, hus corroborating previous data by Shuck and others (1949). By microscopic nd phase-contrast examination of the solutions it was found that the meta-hromatic shifts are brought about by interaction between dye and substrate n a special way. Suspended in the salt-free solutions very thin and mem-raneous micro-particles, probably aggregated micelles, were demonstrated, hich exhibit almost regular patterns of metachromatically red-stained *and* ntercalated orthochromatic areas. After sedimentation, the supernatant solu-on contains excess of dissolved dye with an orthochromatic colour. If the eactions are performed in water containing small amounts of NaCl (0·03 mol) r ethanol (10 per cent.), the micellar aggregates appear stained in blue all over he surface, indicating that metachromasia was hampered.

These observations show that anionic colloidal micelles reach a surface harge density and structural organization sufficient to produce metachro-nasia. The condition is comparable with the results previously mentioned on ubstituted cellulose (Section IV). Further studies of micellar states would eem fruitful (McBain, 1940; see discussions by Corrin, Klevens, and Harkins, 1946).

Lipides. Natural fats and ester phosphatides like lecithins, and also purified holesterol, are largely hydrophobic. Studied as suspensions and at the inter-hases between organic solvents and water, a variety of labile adsorption henomena may be noted. No typical metachromatic reactions have thus been bserved, and the general inability on the part of these compounds to take on he orthochromatic colour suggests that no linkage between the anionic roups of the substrate and the dye molecules occurs. This does not exclude he possibility that under specified conditions highly substituted and/or con-ugated lipides (gluco- and sulpholipides) with a high surface charge density ay show spectral shifts suggesting weak metachromasia (Brante and Nilsson).

Proteins. Sols and gels of extracted and partially purified proteins, such s gelatin, serum albumin and globulin, and also fibrillar proteins such as brin, collagen, keratin, myosin, and silk have all shown orthochromatic

characteristics in reasonably salt-free aqueous suspensions (detailed repo omitted). Labile adsorption phenomena suggesting metachromasia are general seen and easily repelled. In most cases slight spectral shifts to longer wav lengths involving blue-green colours are noted, as described by Sheppard ar Geddes (1945).

VI. Inorganic Compounds

Owing to lack of experimental data, the effects of various anions on dilute sols of cationic dyestuffs are difficult to evaluate. At present only a few empiric

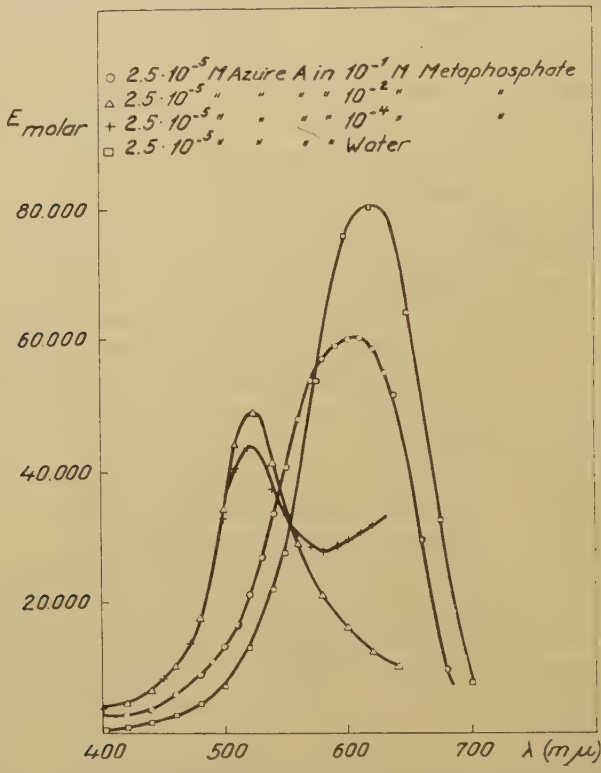


Fig. 13. Absorption spectra of azure A at several concentrations of colloidal synthetic met phosphate.

Substrate No. 1477 (I), in table 8; average particle size 5–10,000. Measurements ma before visual appearance of precipitates. Larger concentrations of substrate than 10^{-2} I inhibit metachromasia. Observations confirm previous data by Wiame on 'hexametapho phate'.

results (table 8) can be discussed in a superficial way, similar to that of Ba and Bungenberg de Jong (1939).

Small anions of the Hofmeister series in general tend to decrease the degr of aggregation of the dye molecules. When the ionic strength is further i creased, a salting-out effect has been noticed as evidenced by *orthochroma* dye precipitates. Two unexplained exceptions were, however, found in t case of I^- and CNS^-, which (in spite of their high 'salting-in' capacity) cau

ronounced *metachromatic* colour shifts and micellar-shaped precipitates (see pek, 1940). Some iso- and hetero-polyacids forming highly hydrated complex xy-salts (see Wells, 1950) with organic compounds also produce metachroatic colour shifts and precipitates with azure A (table 8), probably indicating omplex salt formation.

In the case of inorganic colloidal polyelectrolytes such as metaphosphates Malmgren, 1952) a dye-substrate interaction similar to that of organic polylectrolytes is noted. The degree of metachromasia increases with increasing ggregation and charge density on the part of the substrate. The metachroatic spectral shift in diluted solutions previously described by Wiame (1947) s influenced by the concentration ratio (fig. 13); with increasing substrate concentration and ionic strength the metachromasia is reversed or inhibited.

More information will be obtained when the detailed structure, behaviour o water, and charge distribution on the part of various aggregated anions can e correlated to the formation of such coloured complexes.

VII. Discussion

The available evidence leads to the view that metachromasia is most likely explained as *a special type of orderly dye aggregation characterized by the formation of new intermolecular bonds between adjacent dye molecules*. The particular substrate molecules, mentioned above, may be regarded as centres of orientation, where the free anionic groups will attract the polar groups of the dyestuff (fig. 3) according to the previous hypothesis of Sheppard (1942). Present observations illustrate some essential factors controlling the appearance of metachromasia with organic colloids, namely a high electro-negative *surface charge density*, and *the presence of water*.

Data given in tables 2–6 indicate that *metachromasia requires a certain minimum surface charge density*, viz. a minimum distance between available negative surface charges. In hyaluronic acid, where the distance between the $—COO^{1-}$ groups is about 10·3 Å, no metachromasia appears in the diluted aqueous sol. In three-dimensional lattice gels and films, however, the actual charge over the surface of the particles is quite sufficient to cause this reaction (Sylvén and Malmgren, 1952). Another example is found in partly substituted cellulose fibres which show an irregular 'banded' metachromasia corresponding to the distribution of the $—CH_2.COO^{1-}$ groups, and in which an even distribution of metachromasia is reached when the average degree of substitution exceeds 50 per cent., that is, when at least every second glucose unit of cellulose has become carboxylated (average intercharge distance about 10 Å). In the pectinate molecule, as a straight-chain polymer, the average intercharge distance would be about 5 Å and this is enough to produce a weak metachromasia (fig. 4). Polysaccharides containing per disaccharide unit one $—OSO_3^{2-}$ group plus the uronic acid $—COO^{1-}$ (table 5) are expected to have alternating intercharge distances along the chains of about 4 and 6 Å, and show a moderate degree of rather stable metachromasia. As a result of the

introduction of two or three sulphate groups per disaccharide unit the averag intercharge distance will be less than 4 Å (depending on the location of group and the presence of a remaining —COO^{1-} after substitution is carried out and metachromasia undergoes a marked increase in degree and stability.

In the case of substrate molecules with mixed hydrophobic and hydrophili properties, the presence of large hydrophobic groups probably presents con siderable steric hindrances for metachromatic dye aggregation in non-oriente states. It would appear that in the case of DNA this can be overcome by linea arrangement of substrate molecules and suitable humidity, which alters th inclination of the obstructing purine and pyrimidine bases to the phosphat sugar chain (Seeds, 1953).

The inorganic substrates cursorily investigated may in part form a grou where metachromasia would be better explained by complex formation. Littl is known about salting-out effects, formation of 'ionic micelles', and othe factors possibly of importance.

It seems reasonable to assume that the magnitude of the observed spectra shifts and the relative 'stability' of metachromatic aggregates depend upon th actual bonding energy between adjacent dye molecules. These energy level have been roughly calculated (table 9), but the values obtained give no direc indication as to the actual bonds involved—either several hydrogen bonds or if water molecules were intercalated, hydrogen bonds and perhaps oxygen sulphur bridges (see fig. 3). Since the necessary energies are small with refer ence to biocolloidal substrates, it is possible that 'long-range forces' (van de Waals) may also be involved. It should further be emphasized that the physica conditions and possible types of bonding in metachromasia may be differen both with different dye-stuffs and with different substrates. The metachro matic spectrum of the thiazine dye azure A over a number of compounds is, fo instance, largely similar but not identical with that of the cyanine dye pina cyanol tested in aqueous solution.

A more detailed discussion will be postponed until data are available on the actual intermolecular bond distances in various metachromatic dye aggre gates.

My thanks are due to my friends and co-workers Dr. O. Snellman and Dr H. Malmgren of the Institute of Biochemistry, Uppsala, for helpful criticism and advice, and also to Dr. W. E. Seeds of King's College, London, for dis cussions and linguistic correction of the manuscript. It is further a pleasure to acknowledge the most valuable gifts of many purified and carefully analysed compounds obtained by courtesy of various institutes, investigators, and companies, which have all been mentioned in the tables. The costs have been defrayed by institutional grants from H.M. King Gustaf V's Jubilee Fund, the Swedish Anti-Cancer Society, and the Cancer Society of Stockholm, and also from the Swedish Co-operative Women's Guild, and from the Eli Lilly and Co., Indianapolis, Ind.

TABLE 1

Non-charged carbohydrates devoid of metachromasia to azure A as sols, gels, and films

Substrates	*Source of substrate and remarks on purity*
MONOSACCHARIDES	
Arabinose	Hoffmann–La Roche, recrystallized
Ribose (*d*-)	Same as above
Fucose (*l*-)	Hoffmann–La Roche
Rhamnose	Same as above
Hexoses	Commercial
DISACCHARIDES	
Sucrose, Maltose, Cellobiose, Trehalose, Lactose	High degree of purity (only one spot on paper chromatography).
POLYSACCHARIDES	
Starch (poly-*d*-glucose)	Commercial
Glycogen	Various high grade analytical samples
Dextrans, all available particle sizes investigated	Pharmacia, Inc., Uppsala Some samples contain unknown metachromatic admixtures
Cellulose	Chemical purity
Levulan (poly-fructose)	Bacterial. Dr. Ingelman
Inulin (poly-fructose)	*Dahlia*. Dr. Ingelman
Larch polysaccharide (galactose + arabinose)$_n$	*L. europea*; Mosimann and Svedberg (1942)

TABLE 2

Reactions of some substituted sugars and polysaccharides with a low electro-negative charge

Substrate	*Average degree of substitution*	*Colour reactions of aqueous sols and precipitates*	*Colour stability against adsorption repellants*	*Result as to metachromasia in sol state*	*Metachromasia of conc. solutions, gels, or films*	*Source of substrate*
Glucosamine —HCl	One —NH_2/unit	Orthochromatic blue	..	Negative	Very weak metachromatic shift in films	Hoffmann–La Roche
N-acetylglucosamine	One —$NH.COCH_3$/unit + additional acetyl-groups	,,	..	,,	,,	Laboratory preparation
Glucuronic acid (lactone)	One —COOH/unit	,,	..	,,	,,	Commercial Solvents Corp. and others
Gluconic acid (lactone)	,,	,,	..	,,	Orthochromatic (metachromatic shift with pinacyanol)	Hoffmann–La Roche
Galacturonic acid (free)	,,	,,	..	,,	Orthochromatic	Analytical samples
Na-alginates (polymannuronic acid), high polymers	One —COOH/unit Considerable charge density per particle	Slight metachromatic shift. Precipitates are partly blue and red	Non-stable against salt. Low stability against ethanol	Weak metachromasia	Weak metachromatic shift	Laminal HHF from A/S Protan, Inc., Drammen, Norway
Various pectinates (poly-galacturonic acid), high polymers	One —COOH/unit Actual charge density unknown	,, Cf. fig. 4	Low stability	,,	Gels and films show slight degree of metachromasia	Beet, citrus, and apple pectins purified by Dr. Säverborn (1945)

TABLE 2 (*continued*)

Reactions of some substituted sugars and polysaccharides with a low electro-negative charge

Substrate	*Average degree of substitution*	*Colour reactions of aqueous sols and precipitates*	*Colour stability against adsorption repellants*	*Result as to metachromasia in sol state*	*Metachromasia of conc. solutions, gels, or films*	*Source of substrate*
DEXTRAN SERIES						
Dextran methoxyl ester (—O.CH_3), low particle size	30 per cent.	Orthochromatic solution and precipitates	..	Negative	Weak metachromatic shift	Dr. Ingelman, No. 1237
Dextran carboxymethyl ester (—CH_2.COONa):						
1. Small particle size	Not stated	,,	..	,,	,,	Dr. Ingelman, 4 different samples
2. Medium-sized polymers	,,	Slight metachromatic colour shift; precipitates mainly blue, part is red	Low stability	Part is positive	,,	Dr. Ingelman, Nos. 998 and 1006
Dextran phosphate esters (Na-salt)	P content 20 per cent. (1·8 phosphate groups/period)	Metachromatic colour shift; blue and red precipitates	Gels and films stand 30 per cent. ethanol; sols show low stability	Positive	Marked metachromasia	Dr. Ingelman, Nos. 926 and 926*b*

TABLE 3

Metachromasia depending on charge density referable to sol and gel states of substrate (cf. Sylvén and Malmgren, 1952)

Substrate	*Average degree of substitution*	*Colour reactions and stability*	*Result as to metachromasia*	*Preparation by*
HYALURONIC ACID, high degree of purity, S-free; average particle size about 60,000	One COOH/disaccharide unit	..	..	..
Concentration range (w/vol.):				
0·02–0·6 per cent.	..	Orthochromatic colour and blue precipitates	Negative	Dr. Malmgren, Inst. of Biochemistry, Uppsala
0·6–1·8 per cent.	..	Orthochromatic colour and blue precipitates In places of beginning gel formation some small metachromatic precipitates appear showing low degree of stability	Mainly negative	,,
Above 2 per cent.	..	Increasing amounts of metachromatic membraneous precipitates, presenting moderate stability	Bulk of material negative; *gelated aggregates positive*	,,
Concentrated gels and solid films	..	Metachromatic colour and metachromatic precipitates Metachromatic until 0·09 per cent. NaCl and about 30 per cent. ethanol	Positive	,,
METHYL ESTER OF HYALURONIC ACID, partially degraded, contains mainly —CO.Ch_3	25 per cent.	Purely orthochromatic	Negative as sol, gel, and film	Laboratory preparation

2421.3

TABLE 4. *Microscopic registration of increase in metachromasia in substituted cellulose fibres and gels*

Reactions performed in both suspensions and smears of cellulose, with some excess of dye. Microscopic observations in transmitted light. The increasing degree and locations of substitution also observed in polarized light (see text).

Substrate	*Average degree of substitution*	*Colour reactions and stability*	*Result as to metachromasia*	*Source of substrate*
CELLULOSE	A few COOH groups may normally be present (ref. v. d. Wyk and Studer, 1949). Fibre content 100 per cent.	Orthochromatic fibres and various adsorption phenomena (red-violet-blue, &c.)	Negative	Chemical purity
NA-CARBOXIMETHYL CELLULOSE ($-CH_2.COONa$)				
Sample No. 18 . . .	0·15/glucose unit. Fibre content 93 per cent.	All fibres are orthochromatic, but a few metachromatic spots appear in focal regions	Positive in places of substitution	Research Lab. of Sw. Cellulose Co. Inc.
„ 19 . . .	0·23/glucose unit. Fibre content 71 per cent.			
„ 21 . . .	0·37/glucose unit. Fibre content 2 per cent.	Mainly orthochromatic fibres. In places, stable metachromatic colour appears in increasing degrees	„	„
„ 175 . . .	0·48/glucose unit. Fibre content 2 per cent.			
„ 26 . . .	0·53/glucose unit. Fibre content 1 per cent.			
„ 111 . . .	0·60/glucose unit. Fibre content 1 per cent.	Metachromasia over the whole fibre surface and through gels and fresh and dried films. Stable against 50 per cent. ethanol	Positive	„
„ 113 . . .	0·84/glucose unit. Fibre content 0			
CELLULOSE PHOSPHATE (not investigated)	..	Stain metachromatic	..	Acc. to Lison (1935)
CELLULOSE ESTER SULPHATE (not investigated) . . .	..	„	..	„

Table 5

Polysaccharide ester sulphates showing pronounced metachromasia to azure A at low dye concentrations

Metachromatic spectral shifts and precipitates appear in dilute sols of substrates down to a concentration of about 0·02 per cent.
Degrees of stability refer to concentrations of ethanol, added to sols or gels after the metachromatic interaction has occurred.
All non-substituted original compounds are non-metachromatic (see tables 1–4).
Addendum: Similar observations on sulphate esters of starch, glycogen, cellulose, and pectins previously mentioned by Lison (1935).

Substrate	*Average degree of substitution*	*Metachromasia*	*Stability of metachromasia (ethanol concentration in per cent.)*	*Approximate site of absorption maximum (mμ) in salt-free (?) gels or films*	*Source of substrate*
Na-salt of sulphonated *hyaluronic acid*; partially degraded	2 to 3 S/period	Strongly metachromatic in all concentrations down to 0·02 per cent.	About 50–70	480–500	Mr. Högberg of Leo Research Lab., Inc., Hälsingborg, Sweden
Na-*Chondroitin sulphate*	1 S/period	Low degree of metachromasia, see fig. 5	About 30. Low stability to addition of other ions	540	Prepared from *nucleus pulposus* of young calves
Na-*Heparin* (heterogeneous)	1 to 3 S/period	Strongly metachromatic, see fig. 6	About 50 to 70, see fig. 7	480	Pharmaceutical sample; Vitrum, Inc., Stockholm
Na-*Dextran ester sulphate* (medium particle size)	1 to 2 S/period	Strongly metachromatic	About 50	..	Dr. Ingelman, Nos. 845 and 1001,*c* (Grönwall and others, 1945)
Ca-*Alginic ester sulphates:* Large particle size (heterogeneous)	1 to 2 S/period	One strongly metachromatic and another non-metachromatic component	,,	..	Paritol I. M. Wyeth, Inc., Philadelphia, U.S.A.
Medium particle size	2 to 3 S/period	Metachromatic component predominant	About 50–70	480	Paritol C. Wyeth, Inc., Philadelphia, U.S.A.
Na-*Levulan ester sulphate*	1 to 2 S/period	Strongly metachromatic	About 50	500	Dr. Ingelman, No. 868, *a*
Na-*Inulin ester sulphate*	1 S/period (7–9 per cent.)	,,	About 30	540	Dr. Ingelman, three different samples investigated
Na-*Larch polysaccharide ester sulphate* (inhomogeneous)	1 to 2 S/period	,,	About 40	540	Dr. Ingelman (Mosimann and Svedberg, 1942)

TABLE 6

Salt instability of chondroitin sulphate metachromasia

odium salt of chondroitin sulphate 2 mg./ml. added to 2 × 10^{-5} mol azure A in aqueous lution, temperature 20° C. Mixed red and bluish (violet) precipitates appear in all tubes ter some time, but the amounts decrease with increasing ionic strength of solutions. Abrption spectra give little information as to actual aggregation of dye and substrate (see fig. 5). may be added that under similar conditions a phosphate buffer of 0·04 mol/lit. and a pH of 5·9 is sufficient to suppress metachromatic spectral shifts of chondroitin sulphate.

Medium	$\lambda_{\gamma\ max}$	ϵ_{max}	*Visual colour of solution*
H_2O	590	0·478	Violet
0·003 mol NaCl	600	0·456	Blue
0·03 ,,	605	0·582	,,
0·09 ,,	610	0·600	,,
0·18 ,,	610	0·630	,,
0·3 ,,	610	0·682	,,

TABLE 7

Reactions of ATP *and purified nucleic acids with azure A*

Substrate	*Aqueous solutions*			*Gels and films*	*Result as to metachromasia*	*References*
	Concentrations	*Colours*	*Colour of precipitates*			
Na- and Ca-salts of commercial *adenosine triphosphate* (ATP), further purified; still containing ADP and other low-molecular admixtures. Probably not salt-free	Diluted and concentrated solutions	Orthochromatic. No spectral shift	Bulk of material is orthochromatic. A small admixture of faintly metachromatic material present	..	Negative at all concentrations	For impurities cf. Burrows and others, 1952
Na-salt of RNA, purified from Eastman commercial. Polydisperse, small particle size. Not protein- or salt-free	Solutions of RNA according to legend to fig. 9	Slight colour shift to 570 mμ	At low dye-substrate ratios precipitates are blue-green; at high ratios precipitates become dark blue	Amorphous films are purely orthochromatic at different degrees of hydration	Negative (also with pinacyanol)	See fig. 9 and text
,,	2·5 mg./ml. in NaCl 0·03 mol	Orthochromatic	,,	Orthochromatic (also with pinacyanol)	Negative	..
,,	2·5 mg./ml. in 30 per cent. ethanol	,,	,,	,,	,,	..
Na-salt of DNA, highly purified, large particle size. Residual salt content after electrodialysis about 1–2 per cent.	Concentrations according to legend to fig. 10	Orthochromatic; visual colour blue, no spectral shift	Membraneous precipitates partly metachromatic (see text)	Non-oriented films partly metachromatic (also with pinacyanol). Oriented films strongly metachromatic	Degree of metachromasia depends on molecular orientation	Obtained by courtesy of Dr. G. Frick, Uppsala

TABLE 8. *Some salts exhibiting various degrees of metachromasia to azure A*

Substrates	*Particle size*	*Concentration range*	pH *of solution*	*Colour of aqueous solution*	*Stability*	*Result as to metachromasia*	*Sources and references*
ORTHOPHOSPHATE $K_3.PO_4$	..	Diluted aqueous solution	9–10	Violet colour change. Metachromatic precipitates, until decoloration of solution	Some stability against further increase of ionic strength. Not stable against alcohol and heat	Non-stable metachromasia	Analytical
PYROPHOSPHATE $K_4.P_2O_7$	..	,,	8–9	Orthochromatic colour and metachromatic precipitates	,,	,,	,,
TRIPOLYPHOSPHATE $Na_5.P_3O_{10}$	..	,,	..	Orthochromatic	,,	Negative	,,
METAPHOSPHATES							
A. *Synthetic*, pure, sulphur-free. General formula $(H.PO_3)_n$	..	..	..	..	..	..	Prepared by Dr. Malmgren, Uppsala (ref. Malmgren, 1952)
Na-trimetaphosphate, configuration probably cyclic	306	,,	..	,,	,,	,,	Tf 2
Na-metaphosphate, polydisperse	5–10,000	,,	..	Metachromatic colour of solution and metachromatic precipitates	Stability of metachromasia against salt and alcohol increases with particle size and charge density	Weak metachromasia	1477, I
Na-metaphosphate, polydisperse	15–30,000					..	1477, II
K-metaphosphate	200,000	,,	..	,,	Rather stable	Stable metachromasia	K 17
K-metaphosphate	below 10^6						K 16
K-metaphosphate	10^6	Diluted solution in 0·1 M. NaCl	..	Metachromatic	High stability	,,	K 15
K-metaphosphate	2.10^6						K 21
B. K-metaphosphate isolated from *Asperg. niger*	10–20,000	,,	..	,,	,,	,,	1516, ash content 96 per cent. Impurities present

TABLE 8 (*continued*). *Some salts exhibiting various degrees of metachromasia to azure A*

Substrates	*Particle size*	*Concentration range*	pH *of solution*	*Colour of aqueous solution*	*Stability*	*Result as to metachromasia*	*Sources and references*
IODIDE							
K I	..	Diluted aqueous solution	Neutral	Metachromatic colour shift, with metachromatic precipitates in the form of thin membranes, suggesting micellar complex formation	Rather stable	Stable metachromasia	Analytical
BROMIDE							
KBr	..	,,	,,	Orthochromatic	..	Negative	,,
THIOCYANATE							
K.SCN	..	Aqueous solutions below a concentration of 0·2 per cent.	..	Orthochromatic No precipitates	..	,,	,,
K.SCN	..	Aqueous solutions of concentrations 0·4–5 per cent.	..	Metachromatic colour and micellar precipitates	Stands 30 per cent. ethanol concentration and reappears after heating to 60° C.	Rather stable metachromasia	,,
K.SCN	..	Dissolved in phosphate buffer with ionic strength 0·1	..	No colour change No precipitates	..	Inhibition of metachromasia	,,
TUNGSTATE							
$Na_2.WO_4$	Varies with concentration	Sol and gel states	..	Metachromatic precipitates of micellar type	Stable in alcohol to about 30 per cent.	Rather stable metachromasia	,,
MOLYBDATES							
$Na_2.MoO_4$ and $Am.MoO_4$	..	Diluted aqueous solutions	..	,,	,,	,,	,,
Phospho-molybdic acid (empirical formula $P_2O_5.20MoO_3$)	..	,,	..	Precipitates mainly orthochromatic, in parts weak metachromasia	Low degree of stability	Negative, mainly orthochromatic	,,

TABLE 9

Approximate energy requirements for the production of metachromatic colour shifts

Calculated from Planck's formula; figures expressed as differences from the orthochromatic level of azure A at 615 mμ. Complete metachromasia all over the substrates without admixture of orthochromatic components is first reached when the γ-band prevails; this occurs at about 520 mμ, corresponding to about 8 cal./mol.

Absorption maxima in mμ	$\Delta E = E_{max} - E_{615\ m\mu}$ *in cal./mol*	*Approximate maximum absorption levels of some purified compounds*
600	1	..
580	2·8	RNA, fig. 9
560	4·5	Pectinate, fig. 4
540	6·4	Chondroitin sulphate, fig. 5
520	8·5	KSCN, table 8 Detergents TPBS, fig. 11
500	10·6	Heparin, inhomogeneous; fig. 7
480	13	Trisulphonated hyaluronic acid, table 5
460	15·6	..
440	18·5	..
400	25	..

REFERENCES

Bank, O., and Bungenberg de Jong, H. G., 1939. Protoplasma, **32**, 489.
Brante, G., and Nilsson, S., Personal communication.
Burrows, S., Grylls, F. S. M., and Harrison, J. S., 1952. Nature, **170**, 800.
Carnes, W. H., Weissman, N., and Rubin, P. S., 1951. J. Nat. Cancer Inst., **12**, 240.
Corrin, M. L., Klevens, H. B., and Harkins, W. D., 1946. J. chem. Phys., **14**, 480.
—— and Harkins, W. D., 1947. J. Am. chem. Soc., **69**, 679.
Flax, M. H., and Himes, M. H., 1951. J. Nat. Cancer Inst., **12**, 242.
Frey-Wyssling, A., 1936. Protoplasma, **26**, 45.
Grönwall, A., Ingelman, B., and Mosimann, H., 1945. Uppsala Läkareförens. förhand
Ny följd, **50**: 5–6, 397.
Hansen, Fr. C. C., 1908. Z. wiss. Mikr., **25**, 145.
Herzfeld, S. H., Corrin, M. L., and Harkins, W. D., 1950. J. phys. and colloid. Chem
54, 271.
Holmes, W. C., 1928. J. Am. chem. Soc., **50**, 1989.
Levine, A., and Schubert, M., 1952. Ibid., **74**, 91.
Lison, L., 1935. C.R. Soc. biol., **118**, 821.
—— 1935. Arch. Biol., **46**, 599.
—— and Mutsaars, W., 1950. Quart. J. micr. Sci., **91**, 309.
McBain, J. W., 1940. Nature, **145**, 702.
Malmgren, H., 1952. Acta chem. scandinav., **6**, 1.
Merrill, R. C., and Spencer, R. W., 1948. J. Am. chem. Soc., **70**, 3683.
Michaelis, L., 1926. *Enzyklopädie der mikroskopischen Technik*. Ed. by R. Krause. III. Auflage
Berlin and Wien (Urban & Schwarzenberg), **2**, 1376.
—— 1944. Biol. Bull., **87**, 155.
—— 1947. Cold Spring Harbor Symposia on Quantitative Biology, **12**, 131.
—— 1950. J. phys. and colloid. Chem., **54**, 1.
—— and Granick, S., 1943. J. Am. chem. Soc., **65**, 1747.
—— —— 1944. Ibid., **66**, 1023.
—— —— 1945. J. Am. chem. Soc., **67**, 1212.
Mosimann, H., and Svedberg, T., 1942. Kolloid-Z., **100**, 99.
Ostwald, W., 1933. Ibid., **62**, 365.
Rabinowitch, E., and Epstein, L. F., 1941. J. Am. chem. Soc., **63**, 69.
Robinson, C., 1935. Trans. Faraday Soc., **31**: **1**, 245.
Scheibe, G., 1938. Kolloid-Z., **82**, 1.
Schwarz, R., and Herrmann, E., 1922. Ibid., **31**, 91.
Seeds, W. E., 1953. *Progress in biophysics and biophysical chemistry*. London (Pergamon Press
Ltd.), **3**, 27.
Sheppard, S. E., 1942. Rev. mod. Phys., **14**, 303.
—— and Geddes, A. L., 1945. J. chem. Phys., **13**, 63.
Shuck, G. R., and Lingafelter, E. C., 1949. J. Am. chem. Soc., **71**, 1325.
Spek, J., 1940. Protoplasma, **34**, 533.
Sylvén, B., and Malmgren, H., 1952. Lab. Invest., **1**, 413.
Säverborn, S., 1945. *A contribution to the knowledge of the acid polyuronides*. Uppsala.
Valkó, E., 1935. Trans. Faraday Soc., **31** : **1**, 230.
Wells, A. F., 1950. *Structural inorganic chemistry*. 2nd ed., Oxford (Clarendon Press).
Wiame, J. M., 1947. J. Am. chem. Soc., **69**, 3146.
Wyk, A. van der, and Studer, M., 1949. Helv. chim. Acta, **32**, 1698.

The Histochemical Dephosphorylation of Riboflavin Phosphate (Flavin Mono-nucleotide) and Pyridoxal Phosphate (Co-decarboxylase)

By GEOFFREY H. BOURNE

(*From the Histology Department, London Hospital Medical College, E.1.*)

With four plates (figs. 1–4)

Summary

1. The histochemical dephosphorylation of riboflavin phosphate and pyridoxal hosphate has been studied at pH 9·0 and pH 5·0. The results have been compared ith those obtained with glycerophosphate.

2. It was concluded that these substrates are dephosphorylated by enzymes or nzyme systems differing from each other and from that which dephosphorylates lycerophosphate.

3. The vitamin substrates undergo some dephosphorylation by all tissues examined t pH 9·0. At pH 5·0, however, no reaction is given by any tissue with riboflavin hosphate as a substrate, but all tissues give some reaction at this pH with pyridoxal hosphate.

4. The reaction at both acid and alkaline pHs with pyridoxal phosphate is in some issues located in bodies resembling mitochondria: the sarcosomes of heart-muscle in articular give a strong reaction. This is true at pH 9·0, to a lesser extent, with ribo-lavin phosphate.

5. It is possible that the sites of dephosphorylation shown by the histochemical ests may also be the sites of phosphorylation of these vitamins.

6. Intercalated disks of heart-muscle show an intense dephosphorylating activity or riboflavin phosphate and glycerophosphate.

7. Pyridoxal phosphate and riboflavin phosphate are dephosphorylated at the same ites (differentiating regions) as glycerophosphate in rat embryos.

Introduction

IT has been shown that a number of members of the vitamin B complex in their phosphorylated condition form essential parts of enzyme systems concerned with metabolism. Among these are riboflavin phosphate and pyridoxal phosphate. The first of these is said to form the prosthetic group of the mammalian amino-acid oxidase of Green, and the second is the prosthetic group of several, and possibly all, amino-acid decarboxylases.

In view of the metabolic importance of these substances it was thought that a histochemical investigation of the ability of different tissues to dephosphorylate them would be of importance in establishing (1) whether this is a function of non-specific alkaline phosphatases, (2) if specific phosphatases are responsible, where they are located both from the point of view of the distribution in the various tissues and within the cells themselves.

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 359-369, September 1954.]

There appears to be only one paper on a related subject in the literatu and that refers to the localization in the brain of an enzyme dephosphorylati aneurin pyrophosphate at a neutral pH (Naidoo and Pratt, 1952).

Methods

Male and female rats were the animals used for this work. They were kill by means of a blow on the head and small portions of the following orga were removed and fixed for 24 hours in ice-cold acetone: cerebrum, cer bellum, uterus, kidney, liver, stomach, jejunum, colon, rectum, skelet muscle, heart-muscle, trachea, lung, testis, epididymis, and adrenal. TI ovary was similarly treated but the results obtained with this organ will b published separately in association with R. J. Harrison in a general accou of the histochemistry of the ovary.

After fixation the tissues were embedded in wax and sectioned. They we then incubated in a modified 'Gomori' substrate, after the usual preliminar treatment, as follows:

A. Distilled water	40 ml.
2 per cent. $CaCl_2$	5 ml.
20 per cent. $MgCl_2$	5 drops
Sodium barbital	0·5 grams
Sodium salt of riboflavin, 5′-phosphate	250 mgm.

or pyridoxal phosphate *or* sodium β-glycerophosphat

This gave a mixture at approximately pH 9·0.

B. Distilled water	50 ml.
Molar acetate buffer	2·5 ml.
5 per cent. lead nitrate	1·0 ml.
Sodium salt of riboflavin, 5′-phosphate	250 mgm.

or pyridoxal phosphate *or* sodium β-glycerophosphat

Fig. 1 (plate). A, glycerophosphatase reaction in rat heart-muscle. Only capillaries positiv Higher magnifications show that intercalated disks are also positive.

B, riboflavin phosphatase reaction in rat heart-muscle. Only nuclei of capillary endotheliu positive.

C, riboflavin phosphatase reaction in rat heart-muscle. Higher magnification. Note positi intercalated disks.

D, pyridoxal phosphatase reaction in rat heart-muscle. Positive reaction in fibres.

E, pyridoxal phosphatase reaction in rat heart-muscle. Reaction is granular, indicating th it is sarcosomal in nature.

F, glycerophosphatase reaction in cerebrum of rat. Positive reaction in capillaries.

G, riboflavin phosphatase reaction in cerebrum of rat. Positive pyramidal cells.

H, pyridoxal phosphatase reaction in cerebellum. Positive pyramidal cells.

I, glycerophosphatase reaction in cerebellum of rat. Positive reaction in capillaries.

J, pyridoxal phosphatase reaction in cerebellum of rat. Positive reaction in Purkinje cell

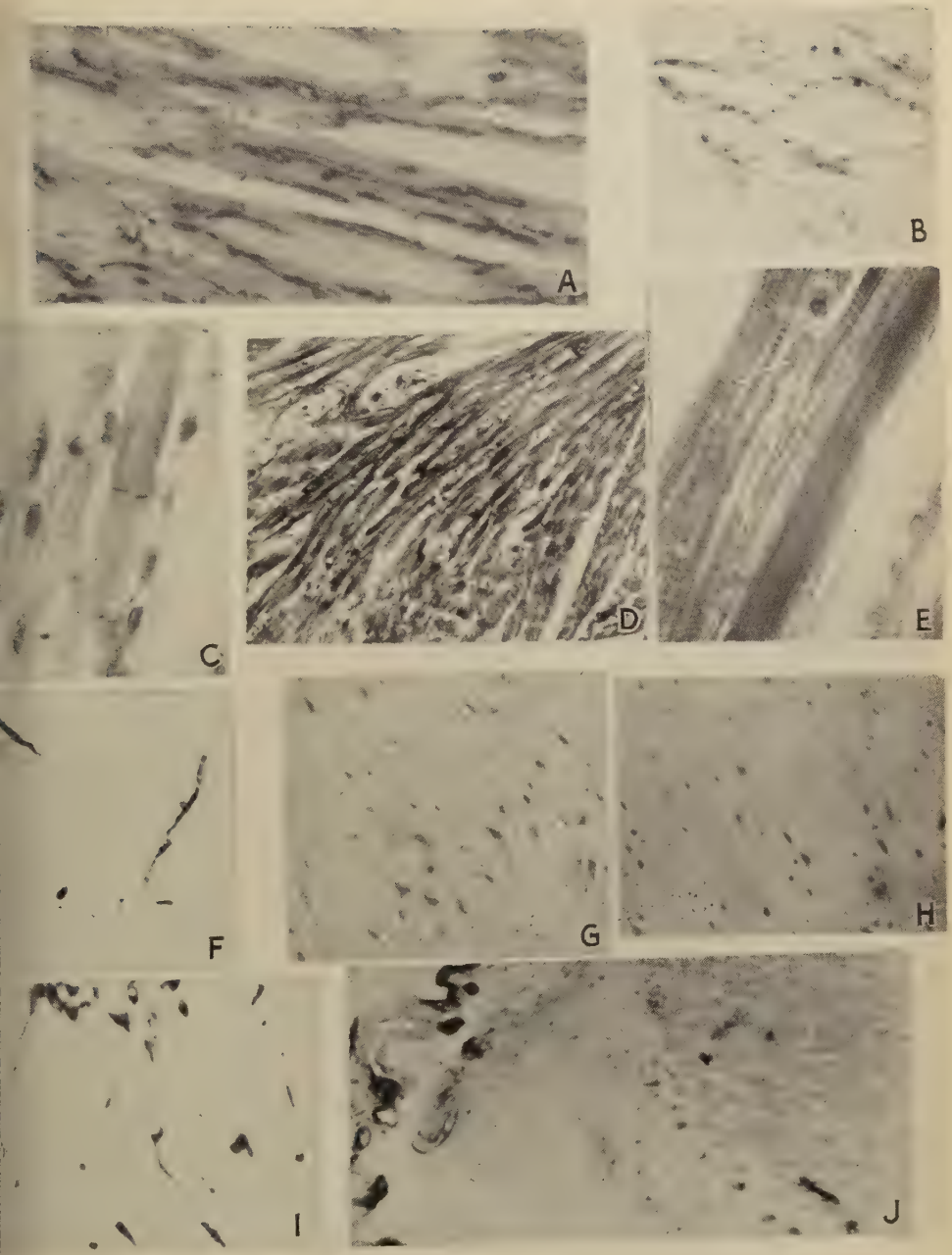


FIG. 1

G. H. BOURNE

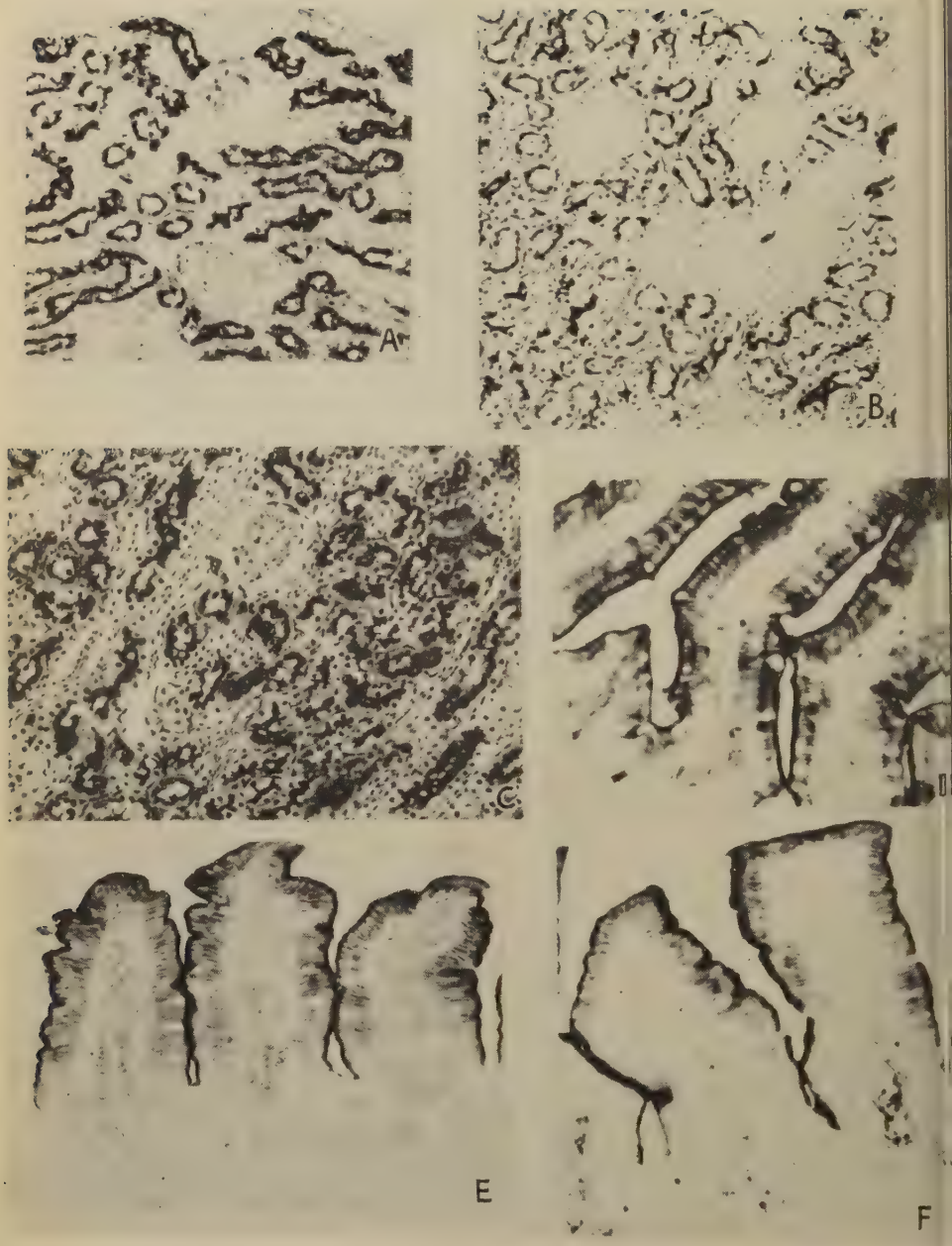


FIG. 2

G. H. BOURNE

There was some precipitate when the riboflavin phosphate was added to the uffer, so that the final concentration of substrate was less than that shown. 'his gave a mixture at approximately pH 5·0.

It is extremely difficult to get completely pure pyridoxal phosphate and ıe sample used was only of 80 per cent. purity.

Incubation was for $2\frac{1}{2}$ hours in the case of substrate *A* and 8 hours in the ase of substrate *B*.

After incubation the sections from substrate *A* were treated as in Gomori's echnique to visualize the results of the reaction for alkaline phosphatase and ı the case of substrate *B* as for Gomori's technique for acid phosphatase.

In addition, sections from the same blocks were used for the demonstration f non-specific alkaline and acid phosphatases by Gomori's methods.

It should be mentioned that, to avoid loss and diffusion of enzyme activity, ections were mounted by simple pressure on the slide instead of by flattening ın water. As a result, particularly intense reactions were given by some organs, .g. free border of the gut, with very short incubations.

Results

Sections incubated at pH 9·0

The results obtained with sections incubated at pH 9·0 are shown in table 1.

Sections incubated at pH 5·0

All tissues incubated with riboflavin phosphate as substrate gave uniformly ıegative results. There was considerable precipitate when the substrate was ıdded to the buffer at this pH, which must have resulted in a much smaller ercentage of the riboflavin phosphate being left in solution. However, even ong incubation failed to give any significant result.

With pyridoxal phosphate many of the cells of the *brain* gave a reaction. In the cerebrum many pyramidal and a number of other cells were positive; in the former, in both nucleus and cytoplasm. Many fibres were also positive. In the cerebellum all cells and some fibres gave some reaction; some Purkinje cells were positive (both nuclei and cytoplasm), some were negative, some were intermediate. Glycerophosphate preparations showed positive capillaries and some cellular reaction.

Fig. 2 (plate). A, glycerophosphatase reaction in rat kidney. Strong reaction in brush borders. Nuclei more or less negative (15 minutes' incubation).

B, riboflavin phosphatase reaction in rat kidney. Weaker reaction in brush border and distinct positive reaction in nuclei (15 minutes' incubation).

C, pyridoxal phosphatase reaction in rat kidney. Strong reaction in brush borders and nuclei (15 minutes' incubation).

D, glycerophosphatase in rat jejunum. Reaction in free borders, in Golgi region, and between the epithelial cells. Very little reaction in nuclei (15 minutes' incubation).

E, riboflavin phosphatase in jejunum of rat. Reaction in free border and between epithelial cells. Slight reaction in nuclei. Golgi region negative (15 minutes' incubation).

F, pyridoxal phosphatase in jejunum of rat. Reaction in free borders and between epithelial cells. Very little reaction in nuclei, reaction in Golgi region in parts. Nuclei negative (15 minutes' incubation).

TABLE 1

Results obtained with sections incubated at pH 9·0

Glycerophosphate pH 9·0	*Riboflavin phosphate* pH 9·0	*Pyridoxal phosphate* pH 9·0
CEREBRUM Capillaries and pia mater positive (fig. 1, F).	Grey matter more positive than white matter. Pia mater and capillaries strongly positive. Three types of positive cell in the cortex: (1) medium-sized cells in which nucleus and cytoplasm gave diffuse positive reaction of equal intensity; (2) pyramidal cells in which nucleus was more positive than cytoplasm; (3) larger cells with positive nucleus, including a very positive nucleolus; cytoplasm negative (fig. 1, G).	Capillaries and pia mater po tive. Pyramidal cells simi reaction to that with ribofla phosphate, deeper cells m positive. Some cells showe positive Golgi area (fig. 1, H)
CEREBELLUM Capillaries and pia mater positive. Nucleoli of some Purkinje and other cells positive (fig. 1, I).	Pia mater and capillaries positive. Everything else negative except intensely positive nucleolus in Purkinje cells.	Grey matter and white mat moderately positive. So Purkinje cells gave a stro positive reaction in both cyt plasm and nuclei, but so were completely negative. O nucleolus positive in some cel in others reaction to one si of cell (that directly towards t surface) (fig. 1, J).
LIVER Positive reaction in parts of bile canaliculi and in bile ducts and in nucleoli of liver cells. Strongly positive cells in sinusoids.	Nucleoli of liver cells nuclei very positive, other parts of cell negative. Strongly positive cells in sinusoids.	A few positive granules in cyt plasm of some liver cel Strongly positive cells in sin soids (fig. 3, F).
KIDNEY Strong reaction in brush borders of proximal convoluted tubule cells. Nuclei negative or faintly positive (fig. 2, A).	Lateral walls of pelvis positive, median parts negative. Large vessels: strongly positive adventitia, negative intima. Intense positive reaction in nucleus and cytoplasm in proximal convoluted tubule cell. Distal one-third of cell and particularly brush borders most positive. All nuclei in cortex and nearly all capillaries positive. Capillaries of glomeruli negative. Medulla negative but collecting tubules showed slight reaction (fig. 2, B).	Both brush borders and nuc more intensely positive th with the other two substra (fig. 2, C).
OESOPHAGUS Capillaries positive.	Capillaries and lymphoid tissue positive. All else negative.	Capillaries and lymphoid tis positive.

TABLE I (*continued*)

Results obtained with sections incubated at pH 9·0

Glycerophosphate pH 9·0	*Riboflavin phosphate* pH 9·0	*Pyridoxal phosphate* pH 9·0
TOMACH apillaries positive.	Capillaries and lymphoid tissue positive. All else negative.	Mucous cells diffusely positive. All other cells show some positive granules. Peptic cells fairly strong positive. Smooth muscle positive.
EJUNUM trongly positive distal border o cell., particularly free border. Golgi apparatus positive. Nuclei negative. Intercellular substance between epithelial cells positive (fig. 2, D).	Free borders of epithelial cells of villi strongly positive. Reaction decreased in crypts, only nucleoli positive in deeper parts of crypts. Negative Golgi apparatus. In some cells a positive mitochondrial reaction was present. Intercellular substance between epithelial cells positive (fig. 2, E).	Free border and nucleoli of epithelial cells of villi positive. Nuclei in crypts cells positive. Smooth muscle positive. Some Golgi regions positive. Intercellular substance between cells positive (fig. 2, F).
COLON Capillaries, nuclei of crypt cells (particularly nucleoli), and adventitia of large vessels positive. Contents positive.	Adventitia of small blood-vessels positive, intima negative. Deepest cells of crypts showed positive cytoplasmic granules. Connective tissue cells and lymphocytes between crypts showed positive nuclei and positive cytoplasmic granules in some cells. Cells of Meissner's nerve plexus appeared positive. Contents positive.	Nuclei of epithelial cells, smooth muscle, and connective tissue between crypts positive.
RECTUM Same as for colon.	Rectum negative except for occasional positive leucocytes and isolated patches of positive connective tissue cells. Contents positive.	Rectum similar to colon.
HEART-MUSCLE Positive capillaries, adventitia of coronary vessels, and intercalated disks. Only capillaries demonstrated in this figure (fig. 1, A).	Nuclei of heart-muscle cells, capillaries, adventitia of coronary vessels, and fibres slightly positive. This reaction was mainly in sarcosomes, though a strong positive reaction was given by the intercalated disks (fig. 1, B and C).	Strong positive reaction by sarcosomes. Slight reaction by smaller vessels, but adventitia of larger vessels positive (fig. 1, D and E).
TRACHEA Similar to riboflavin phosphate.	Ciliated and basal cells and capillaries positive; also nuclei, cytoplasm, cilia, and basal granules.	Similar to riboflavin phosphate.

TABLE 1 (*continued*)

Results obtained with sections incubated at pH 9·0

Glycerophosphate pH 9·0	*Riboflavin phosphate* pH 9·0	*Pyridoxal phosphate* pH 9·0
LUNG		
Similar to riboflavin phosphate.	Bronchial epithelial cells (nucleus and cytoplasm) positive. Alveolar cells and capillaries negative. Leucocytes in vessels and alveolar phagocytes positive.	Similar to riboflavin phospha but bronchial epitheliu stronger reaction, margins alveolar cells positive.
ADRENAL		
All sinusoids in cortex positive. Cytoplasm of all cells negative, nuclei of all cortical cells positive. Medulla negative. Capsule negative (fig. 3, G).	Medulla negative. In cortex, capsule negative, zona glomerulosa intensely positive (fig. 3, H). Cells of zona glomerulosa showed positive nuclei, diffuse positive cytoplasm, and positive mitochondria. In the fasciculate and reticulate zones only the nuclei positive. Sinusoids mostly negative.	Strong positive reaction nucleus and cytoplasm. Mo intense in zona glomerulos Cytoplasm—diffuse reactio also mitochondria appear pos tive. Nucleoli, cell, and nuclear mem branes positive. Light and da reacting cells in zona ret cularis. Medullary cells—cyt plasm negative except for few slightly positive granule nuclei and nucleoli moderate positive. Capsule negative (fi 3, I).
TESTIS		
Leydig cells negative. Capillaries and small blood-vessels, basement membranes of seminiferous tubules and nuclei of basal cells of tubules positive (fig. 3, A).	Leydig cells (particularly nuclei), capillaries, and basal cells of tubules positive. Other spermatogenetic cells slightly positive and sperms negative, basement membranes of seminiferous tubules only slightly positive (fig. 3, B).	Similar to riboflavin PO_4, bu all spermatogenetic cells gav reaction in nucleus and cyt plasm. Latter reaction diffus and granular. Sperms negati (fig. 3, D).
EPIDIDYMIS		
Basement membranes of tubules, small blood-vessels, and capillaries, cilia, nuclei, and nucleoli of epithelial cells positive. Large blood-vessels showed positive adventitia.	Negative except for a positive reaction in some strands of connective tissue lying between tubules.	Nuclei, cytoplasm, and cilia epithelial cells general diffus positive reaction. A few dar granules in the cytoplasm con tents positive (fig. 3, C).
UTERUS		
Similar to riboflavin phosphate.	Capillaries, connective tissue nuclei, and epithelial cells (particularly nuclei and distal edges) positive.	Small arteries and veins nega tive, capillaries, connectiv tissue cells round lumen, an epithelial cells (nuclei, nucleol and general cytoplasm, parti cularly apical portion) positive

n the *liver* and *cortex of kidney*, with pyridoxal phosphate, there was a ht diffuse reaction in both cytoplasm and nuclei and in a few small, posi-, cytoplasmic granules. With glycerophosphate, liver cells and bile ducts re strongly positive. In the cells, the nuclei and what appeared to be mito-ndria gave a reaction. In the kidney, glomeruli (nuclei and cytoplasm), ximal convoluted tubules (nucleus and mitochondria-like bodies), nuclei Henle's loop, and collecting tubule cells were positive.

Skeletal- and *heart-muscle* both gave a strong positive reaction with pyri-xal phosphate; in the former it appeared more intense in the fibrils (parti-larly in the *A* disks). In heart-muscle it was strongest in the sarcosomes. ith glycerophosphate both kinds of muscle were negative.

In the *adrenal* with pyridoxal phosphate the capsule and mature fat cells sociated with it were negative; immature fat cells were moderately positive, myelinated nerve fibres near the gland also were positive. Medullary and rtical cells showed positive nuclei and mitochondria. With glycerophosphate e nuclei of both medulla and cortex were positive, the cytoplasm of all rtical cells was negative; in the medulla, the cytoplasm of some cells was sitive.

In the *trachea* and *lungs* the two substrates gave a similar reaction. In the rmer the cilia and basal granules of the ciliated cells were positive and posi-ve granules were also found in the cytoplasm. The basal cells, however, gave stronger reaction than the ciliated cells and the nuclei of the connective ssue cells were also positive. Only a slight reaction was given by the lung, t the bronchi were similar to the trachea.

In the *testis* a similar reaction was also given by the two substrates. All permatogenic and Leydig and most Sertoli cells were positive. With pyri-oxal phosphate, however, the inner (more mature) spermatogenic cells gave ne strongest reaction (fig. 3, E). The nuclei of the cells and cytoplasmic ranules resembling mitochondria with both substrates gave an equal reaction. he heads of the sperms were also moderately positive. In the epididymis ark granules were seen, particularly in the distal part of the cytoplasm of he epithelial cells. The nuclei were also positive.

Uterus. With both substrates a slight positive reaction was given by the pithelium and the glands, but only a moderate reaction was given by the mooth muscle with glycerophosphate, whereas a strong one was given with yridoxal phosphate.

Alimentary tract. The epithelium of the oesophagus was moderately posi-ive using pyridoxal phosphate. There was a line of positive granules and cells t the junction with the keratinized layer. In the stomach only a very slight eaction was given by the mucosa. The muscle layer and Auerbach's nerve lexus gave a moderate positive reaction. In the jejunum the villi were trongly positive, particularly the epithelial cells, where in the cytoplasm a umber of positive threads and granules resembled mitochondria. The con-ents of the lumen were also positive. The free border was positive, but there was an area just below it in each cell which was even more positive: this was

probably the Golgi apparatus. The nuclei and cytoplasm of cells in the cry
were also positive and so were the smooth muscle coats. In the large intest
the muscle layers and Auerbach's plexus were both positive, but the mus
nuclei were negative. The muscularis mucosae and the smooth muscle of t
blood-vessels also gave a reaction and so did the surface epithelium and cryp
in the cells it was partly diffuse and partly granular.

With glycerophosphate the nuclei of the epithelial cells from the oesopha
to rectum were positive, but with the exception of the jejunum the cytopla
of the cells was not very positive. The reaction in the epithelial cells of t
mucosa was similar to that with pyridoxal phosphate.

Embryonic tissues

Rossi and others (1951 *a* and *b*) have shown that alkaline glycerophospha
is present in the developing human embryo. They found that the enzyme w
associated with ontogenetic processes and was particularly active in t
developing neural tube, acoustic vesicles, optic cups, bones, epithelium of t
alimentary canal, mesonephros, metanephros, germinative area, pulmona
rudiments, and myomeres, and also around arteries.

Alkaline glycerophosphatase preparations of embryonic rats (7 and 15 mm
in the present work showed a similar distribution of activity to that record
by the above authors (fig. 4, A and D). When riboflavin phosphate and pyr
doxine phosphate were used as substrates (also at pH 9·0), it was found th
the distribution of the reaction was exactly the same as for glycerophospha
(fig. 4, B and E). The localization of phosphatases in identical sites is pr
sumably associated with the synthesis of a wide variety of biological sub
stances taking place there. It was of interest that when pyridoxine phospha
was used the reaction at the above sites was more intense than with any of th
other substrates (fig. 4, C).

In the younger rat embryos phosphatase activity was found in developin

---

FIG. 3 (plate). A, glycerophosphatase reaction in testis of rat. Positive reaction by bas
membrane of seminiferous tubules and capillaries. Peripheral cells (spermatogonia) give
slight reaction.

B, riboflavin phosphatase reaction in testis of rat. Virtually no reaction by basal membran
but nuclear reaction by spermatogonia and spermatocytes. Former more active than latte
A positive nuclear reaction can also be seen in the interstitial cells.

C, pyridoxal phosphatase in epididymis of rat. Contents moderately positive; cells an
bunches of cilia positive.

D, pyridoxal phosphatase (alkaline) in testis of rat. Strong positive reaction by basal mem
brane and by all sperm-producing cells and by capillaries and interstitial cells.

E, pyridoxal phosphatase (acid) in testis of rat. Positive reaction given by mature sperm
producing elements.

F, pyridoxal phosphatase in liver. Only a slight reaction by hepatic cells. Strong reactio
by nuclei of cells in sinusoids, which appear to be leucocytes.

G, glycerophosphatase in adrenal cortex of rat. Positive reaction in sinusoids and nuclei

H, riboflavin phosphatase in adrenal cortex of rat. Strong nuclear reaction, particularly i
zona glomerulosa. Sinusoids negative.

I, pyridoxal phosphatase in adrenal cortex of rat. Positive nuclear and cytoplasmic reactio
particularly in zona glomerulosa. Sinusoids negative.

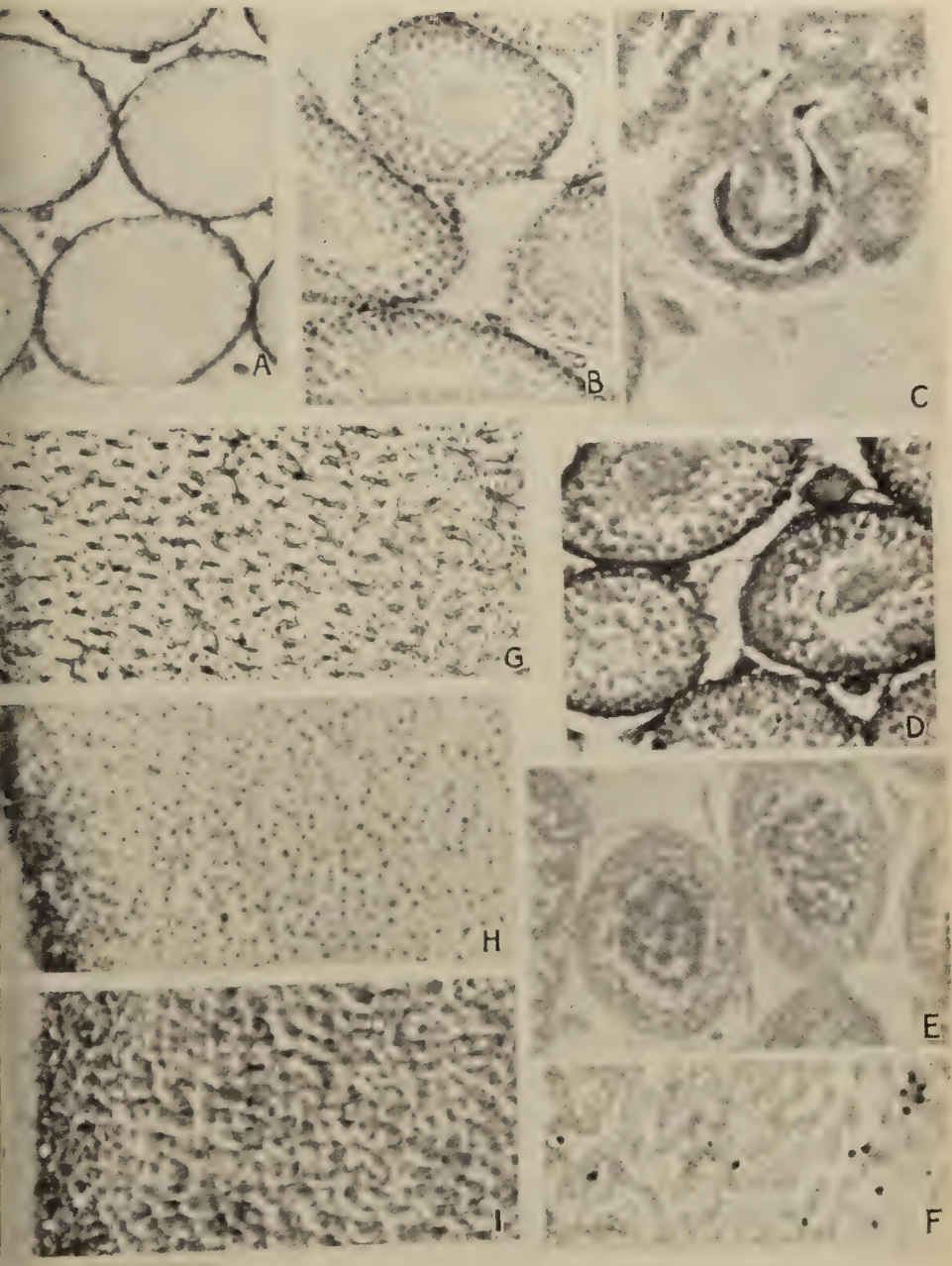


FIG. 3

G. H. BOURNE

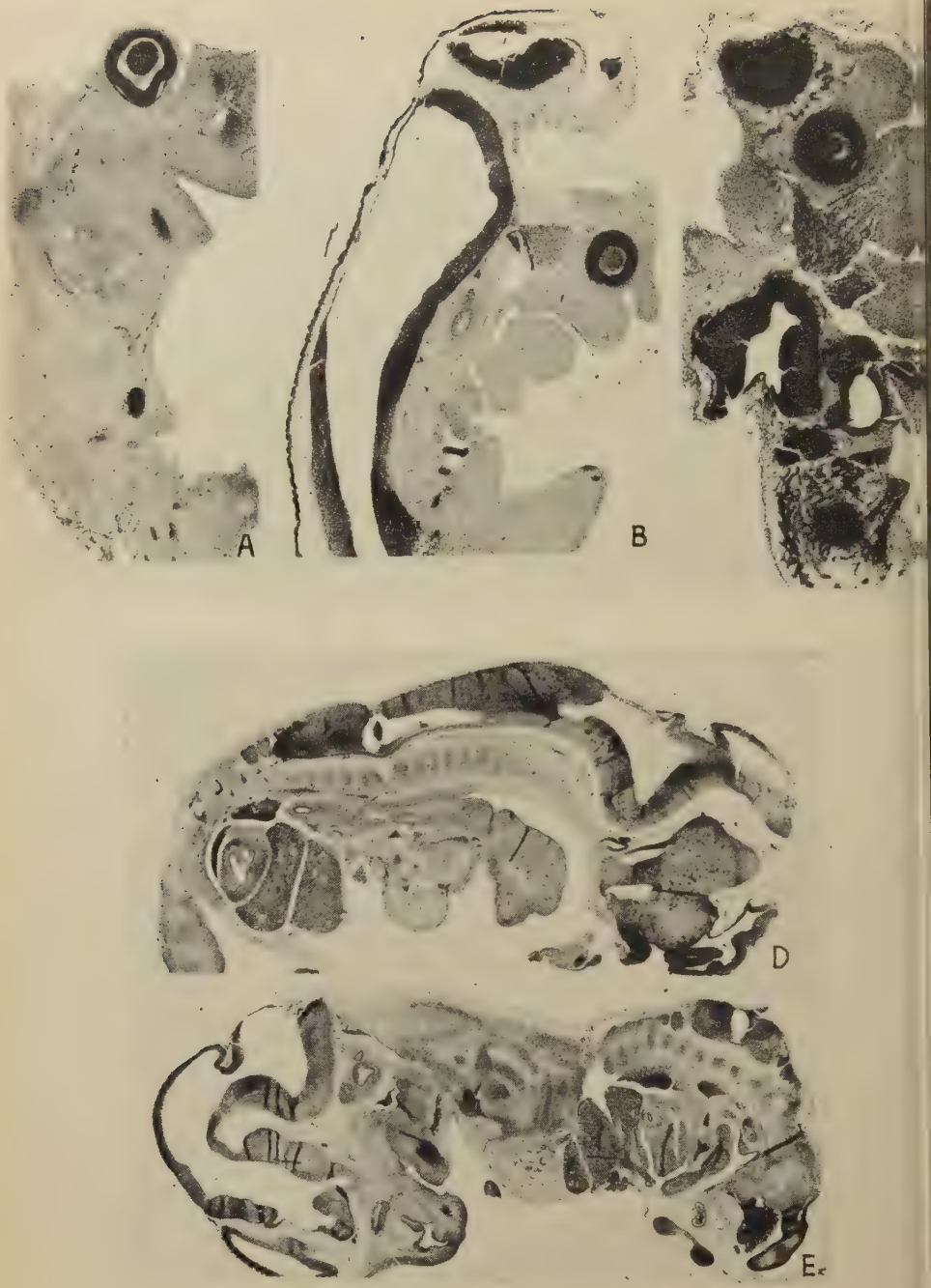


FIG. 4

G. H. BOURNE

rvous system (brain, spinal cord, ganglia, and nerves), optic cup (and lens), c vesicle, bones, gut, muscle, and liver, kidneys, and lungs. In the older rat lbryos the developing blood-vessels, especially the capillaries, were strongly sitive with all substrates. The upper part of the gut became negative, but the ferentiating spleen, liver, and adrenal were all strongly positive. The brain d spinal cord were still positive, but were less so than with younger embryos. the younger embryos the nuclei of the cells were strongly positive with all bstrates, the greatest reaction being given with pyridoxine phosphate.

Discussion

The first problem to be discussed is whether non-specific phosphatases are sponsible for the dephosphorylation of these substrates. A consideration of e results suggests that this is not so. Dealing first with the results obtained an alkaline pH; the brain with glycerophosphate shows negative cells, sitive capillaries, and positive pia mater. The two vitamin substrates show positive pia mater but either negative or only slightly positive capillaries and me positive cells. On the other hand, liver and kidney give fairly similar sults with all the substrates used, although in the kidney a stronger reaction as given by the nuclei with the two vitamin substrates. In the stomach actions were given by the epithelial cells with pyridoxal and riboflavin phosnates, but glycerophosphate gave positive results only in the capillaries with erything else negative. In the small intestine the free borders were positive ith all three substrates, but whereas the Golgi apparatus was positive with ycerophosphate it was positive with only one of the vitamin phosphates. A fference was seen between the vitamin phosphates and glycerophosphate in eart-muscle; with both the former the capillaries were almost completely egative, but with pyridoxal phosphate the sarcosomes were positive. With ycerophosphate the capillaries gave a strong positive reaction but everyning else was negative. In the adrenal an unequivocal difference in reaction etween the vitamin phosphates and glycerophosphate was again detectable. Vith uterus and with trachea and lungs the results with the various sub-

---

Fig. 4 (plate). A, glycerophosphatase in 7 mm. rat embryo, showing high concentration of nzyme activity in developing lens and optic cup. Section does not pass through developing ervous system, but this also shows considerable enzyme activity.

B, riboflavin phosphatase activity in 7 mm. rat embryo, showing concentration of enzyme tivity in developing nervous system, optic cup, and lens.

C, pyridoxal phosphatase in 7 mm. rat embryo. Very high enzyme activity is seen in eveloping nervous system and eye elements, also in developing otic capsule. In lower portion f photomicrograph the localization of the enzymes in the network of developing muscle can e seen. All nuclei in the preparation give a very intense reaction.

D, glycerophosphatase preparation of 15 mm. rat embryo. What appears to be a fine prepitate on the preparation actually represents developing blood-vessels. The brain gives a oderate positive reaction. Developing spleen and adrenal are strongly positive and developg liver moderately positive.

E, riboflavin phosphatase of 15 mm. rat embryo. Moderate reaction in developing nervous ystem and liver. Strong reaction in developing adrenal. No special reaction given by developg blood-vessels.

strates were more or less the same. There were differences also with testis, e the Leydig cells were positive with the vitamin phosphates and negative w glycerophosphate; they gave a very strong reaction with pyridoxal phospha The differences in reaction in the different organs is clearly seen in t illustrations.

It seems that, although there is some coincidence of distribution in so organs, there are sufficient differences to justify the assumption that t vitamin phosphates are not, in these preparations, being dephosphorylat by non-specific phosphatases.

Another reaction which needs to be considered is whether the vitam phosphates are themselves dephosphorylated by a common enzyme. T results obtained with riboflavin phosphate and pyridoxal phosphate a similar in some organs, but, for example, the reaction of heart-muscle with t former is very different from that obtained with the latter, so that it see probable that the riboflavin phosphate and pyridoxal phosphate are al hydrolysed by different enzymes.

What is the significance of the presence in the body of enzymes capable dephosphorylating these vitamin phosphates? It seems likely that we are dea ing here with a phosphorylating/dephosphorylating or transphosphorylati system which may be concerned with the process of synthesis of the phosphat of these vitamins and that under the conditions described in this paper t system dephosphorylates.

At an acid pH (5) riboflavin is not dephosphorylated at all: with pyridox phosphate, however, dephosphorylation was obtained in brain, liver, kidne stomach, small intestine, heart-muscle, testis, adrenal, and uterus.

In most of these organs, where a positive reaction occurred in the cytoplas with pyridoxal phosphate at an acid pH, it was localized in small bodies whic resemble mitochondria in a number of cells. This is particularly well show by the heart-muscle where the sarcosomes, which are mitochondrial in orig and nature, gave a strong positive reaction. A similar reaction was also give by the sarcosomes with this substrate at an alkaline pH. In addition, th tendency for bodies resembling mitochondria to give a positive reaction shown in a number of organs including heart-muscle when riboflavin pho phate is used as a substrate, but this reaction is not so strong as with pyridox phosphate. Reactions in bodies resembling mitochondria were also given b pyridoxal phosphate in alkaline substrate mixtures in, e.g., adrenal. Nuclea reactions are also present with both vitamin substrates: nucleoli in particula give a strong reaction at an alkaline pH. In some organs this may be a dif fusion artifact, but in others, as in the cerebrum, the nuclei of some cells a strongly positive but the cytoplasm completely negative. It seems hard likely in this instance that the nuclear reaction is a diffusion artifact. Nuclea membrane reactions were also present in some organs.

The accumulation of enzymes dephosphorylating the vitamin substrates i differentiating regions of developing embryos, which are presumably regio where a number of chemical substances are being synthesized, is probabl

nificant. It is of interest that in the embryos so far investigated the dis-
bution of enzymes dephosphorylating riboflavin phosphate and pyridoxal
osphate have an identical distribution with non-specific phosphatase.

I am greatly indebted to Dr. A. L. Morrison, Director of Research of
oche Products, Ltd., Welwyn Garden City, for a generous supply of the
tamin phosphates used in this work.
This work was facilitated by a grant for microscopical equipment provided
the Central Research Fund of the University of London.

REFERENCES

AIDOO, D., and PRATT, O. E., 1952. J. Neurol. Neurosurg. Psychiat., **15**, 164.
OSSI, F., PESCETTO, G., and REALE, E., 1951*a*. Zeit. Anat. Entwick., **115**, 500.
—— —— —— 1951*b*. Ibid., **116**, 190.

ne Epicuticle of an Arachnid, *Palamneus swammerdami*

By G. KRISHNAN, Ph.D.

(*From the Department of Zoology, University of Madras*)

With one plate (fig. 2)

Summary

The epicuticle of *Palamneus swammerdami* in the unhardened condition is homo-neous. It stains uniformly red with Mallory. When hardened, it comprises an internal nstained region, giving evidence of —S—S— bonding, and is bounded externally by very thin membrane which stains blue with Mallory.

The protein of the inner basal layer differs from its counterpart in the insect epicu-cle in the absence of tyrosine, in the occurrence of cystine and cysteine, and in being sistant to the action of hot alkalis. Though apparently related to the keratin of ertebrates, it is not identical with it. The outer thin membrane is lipide in nature. -ray diffraction studies show that the epicuticular protein is unique: it is unlike oth arthropodin and the keratin of vertebrates. However, the outer membrane of the picuticle yields a diffraction pattern indicating the presence of long-chain paraffins nd is similar to the outer epicuticle of the blowfly larva. The chitin of the cuticle ppears to be identical in pattern with that of insects.

In spite of differences in structural and chemical constitution, the epicuticle of the corpion shows a resemblance to the basic pattern of the insect epicuticle. The differ-nces may be attributed to the absence of phenolic tanning and the occurrence of —S—S— bonding. The possible role of the purines present in the cuticle of the corpion is discussed in relation to —S—S— bonding.

Contents

Introduction

In a recent study Dennell and Malek (1953) pointed out that the insect epicuticle, notwithstanding the different views put forward regarding its constitution, conforms to a basic pattern comprising an inner and outer layer, the former homologous with the inner epicuticle of the larva of *Sarcophaga* (Dennell, 1946) and the cuticulin layer of *Rhodnius* (Wigglesworth, 1947), while the latter corresponds to the paraffin epicuticle of *Sarcophaga* and the

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 371-381, September 1954.]

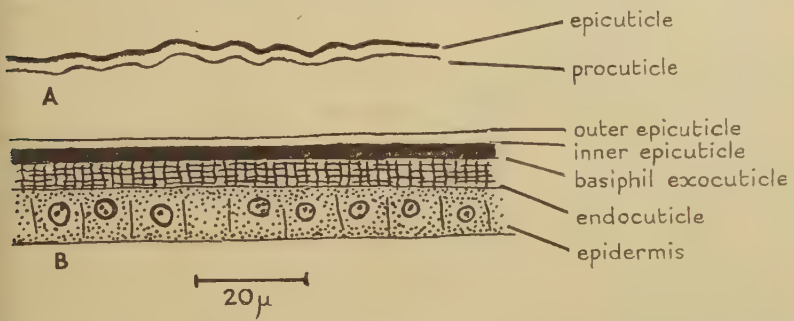
outlying resistant zone of the cuticulin layer in *Rhodnius*. These two layers said to constitute the fundamental parts of the insect epicuticle, while more external ones such as the polyphenol and cement layers appear to variable in their occurrence and probably formed as a result of the elaborati and specialization of the basic pattern. It is not known whether a simi homology can be extended to other classes of Arthropoda. The cuticle arthropods other than insects has received comparatively little attention. Crustacea, Dennell (1947) and Krishnan (1951) described two layers in t epicuticle, apparently corresponding to the outer and inner epicuticle of t larva of *Sarcophaga*, but no attempt has been made to establish the homolo of these layers with those of the insect epicuticle. In myriapods very little known of the constitution of the epicuticle, although Langner (1937) a Cloudsley-Thompson (1950) described a colourless epicuticle in this grou In the Arachnida the information regarding the epicuticle is meagre and co tradictory. Browning (1942) observed that an epicuticle is absent in *Tegenari* but Sewell (1951) who studied the same genus affirmed its presence. In t ticks, Lees (1946, 1947) described an epicuticle identical with that noted Wigglesworth in *Rhodnius*, whereas in *Palamneus* it has been found that t cuticle is very different from that of insects in its structural and chemic features (Krishnan, 1953). It was therefore thought desirable to examine greater detail the epicuticle of an arachnid such as *Palamneus* to find how f it is in accord with fundamental pattern of the epicuticle suggested for insect

The cuticle in different growth-stages of the scorpion *Palamneus swamme dami* was studied by examination of hand, frozen, and paraffin sections. T technique employed in the preparation of the sections is the same as describe in a previous communication (Krishnan, 1953). The chemical reagents use for histochemical tests are mentioned appropriately in the text. X-ray diffra tion studies were made in an attempt to investigate the molecular configur tion of the constituent layers of the cuticle and the nature of the substan orientated in them. For this purpose preparations of the cuticle containing on or the other of the principal constituent layers were made and each of the was stuck to a glass slide over a pin hole made in it, through which the X-ra beam was passed. In the case of the epicuticle several of the layers we superposed on each other so as to increase the bulk of material and thu facilitate a better definition of the X-ray pictures obtained from them. X-ra photographs were taken by using Cu $K\alpha$ radiation at distances of 4 cm. an 5 cm. from object to film.

The Epicuticle

The epicuticle of the scorpion, like that of a number of other arthropod can be separated from the underlying layers by treatment with chlorate nitric acid (see Cloudsley-Thompson, 1950). A similar separation from th rest of the cuticle may also be obtained by treatment with hot concentrate caustic potash. In material prepared for the chitosan test the epicuticle is see as a thin colourless layer separated from the exocuticle. From the negativ

ction to chitosan test it may be inferred that it is non-chitinous, as in ects. In the unhardened cuticle such as is found in the arthrodial membrane juvenile specimens, the epicuticle is undifferentiated; it stains uniformly with Mallory and blue with haematoxylin (fig. 1, A). In an epicuticle which undergone hardening, as in the tergites of the adults, two distinct regions be made out, an inner comparatively wide zone which is not reactive to any the commonly used stains and a very thin outer bounding membrane which ins blue in Mallory (fig. 1, B). Examination of the cuticle in different owth-stages shows that as in insects the hardening of the inner region results a loss of staining property. However, the reactivity to stains can be restored



G. 1. Transverse section through the cuticle of *Palamneus* (a very young specimen). Stained by Mallory's method. A, arthrodial membrane. B, tergite.

y treatment with alkaline sodium sulphide, which, as is well known, has the ffect of breaking up —S—S— bonds. After such treatment, the previously nstained region takes up a red colour with Mallory, while the bounding iembrane still stains blue. In thus consisting of two layers staining blue and ed with Mallory, the epicuticle of scorpion recalls strongly that of the larva f *Sarcophaga* and *Calliphora*. But unlike these, there is no evidence of phenolic anning, as may be inferred from the absence of an amber coloration as well as rom the negative reactions to tests for phenolic compounds.

The inner epicuticle

The inner layer, which may vary in width in different regions of the body rom about 1 to 5μ, undergoes hardening by —S—S— bonding very early in ts growth (see Krishnan, 1953). The epicuticle of the scorpion differs from its counterpart in insects not only in the occurrence of —S—S— bonding but lso in the absence of hardening by phenolic tanning. Correlated with this eature the protein constituents of the layer appear to be different from those f the insect epicuticle. This is suggested by the negative xanthoproteic and Millon's tests. On the other hand, the alkaline lead acetate test is positive, ndicating the presence of cystine or cysteine sulphur. The occurrence of —SH groups is specially indicated in the outer region by the sodium nitro-prusside reaction yielding a purple colour. A similar colour reaction is also

obtained with acetone. The above tests together with the observation of transitory blue coloration in this region on treatment with warm sulphuric acid may show that in a fairly hardened epicuticle, sulphydryl groups are concentrated in the outer zone. A prominent constituent of the epicuticular protein appears to be arginine, indicated by positive arginine tests. A carmine colour is developed on treatment with 2 per cent. alcoholic α-naphthol followed by addition of bromine water. The presence of organic sulphur and arginine

TABLE 1

Name of the test	*outer epicuticle*	*inner epicuticle*
conc. nitric acid (solubility in)	—	—
conc. sulphuric acid (solubility in)	—	—
conc. hydrochloric acid (solubility in)	—	—
conc. sodium or potassium hydroxide (solubility in)	—	—
acetic carbol Sudan test	+++	+++
xanthoproteic	—	—
Millon's	—	—
alkaline lead acetate test	—	+++
arginine test	—	+++
sodium nitroprusside test	—	++
toluene (solubility in)	+	— —
benzene (solubility in)	+	— — —
argentaffin test	++	+++
Fehling's test	+	++
murexide test	+	+
chitosan test	—	—
ferric chloride	—	—
Morner's reagent	—	—

together with resistant properties shown by the protein of the epicuticle (table 1), are suggestive of its relationship to keratin. Further support for this view is afforded by the softening effect on the epicuticle produced by alkaline sodium sulphide, for it is well known that keratins are attacked by alkaline metallic sulphides. But unlike true keratins which are dissolved by concentrated alkalis, the sulphur-linked protein of the epicuticle is resistant to the action of concentrated potassium hydroxide even when heated to 180° C. for several hours. However, the above-mentioned properties are suggestive of a close relationship to scleroproteins hardened by sulphur linkages.

As is well known in the insects, in addition to protein the epicuticle contains lipides. The outer zone of the basal layer is marked by a concentration of lipides, which is shown by an intense sudanophil reaction. With the acetic carbol Sudan III method of Jackson (see Glick, 1949), which is known to reveal the more refractory lipides, the entire epicuticle stained a diffuse red, the narrow outermost zone taking up a more intense red colour. It may be inferred that the lipides of the epicuticle are not confined exclusively to the outermost layer but appear to permeate the epicuticle in varying degrees of concentration.

he outer epicuticle

The epicuticle is bounded externally by a very thin membrane less than
ι thick and distinguishable from the rest of the epicuticle by its staining blue
ith Mallory stain, in contrast to the red-staining inner layer. In its position
ıd staining reactions it recalls the outer epicuticle of the *Sarcophaga* larva.
rom table 1, which summarizes the results of the chemical tests on this layer,
is seen that this layer shows resistant properties, being indifferent to the
tion of hot concentrated acids and alkalis. Pieces of hardened cuticle treated
ith KOH at 180° C. for 2 hours and subsequently sectioned still showed the
ıembrane. But sections prepared from material subjected to hot KOH for
ng periods did not show this membrane. Presumably it is displaced and lost
the course of the preparation. Although concentrated HCl does not dissolve
is layer, yet it was seen that after a preliminary treatment with this reagent
r 2 days, when the cuticle was sectioned and stained with Mallory, the
ıembrane in question took up a red colour instead of the blue seen in normal
ctions. The significance of such a reversal of staining reaction is not clear,
though it may appear that the layer or some of its constituents may be
ısceptible to the above treatment. It is of interest to note in this connexion
ıat a similar reversal of staining reaction has been obtained when the epicu-
cle is subjected to the murexide test and subsequently sectioned and stained
ith Mallory. Treatment with concentrated nitric and sulphuric acids did
ot affect the membrane. That the chemical constitution of this layer is
ifferent from that of the inner layer is evident from the absence of any effect
n this membrane by treatment with alkaline sodium sulphide and other
sts for sulphur-bonded scleroproteins, although it is positive to tests for
pides. In the above chemical features as well as in the staining reactions, a
ery close similarity is observable to the outer epicuticle of *Sarcophaga* and
alliphora. In the blowfly larva, Dennell and Malek (1953) have shown that
ıe outer epicuticle is formed of long-chain paraffins. It remains to be seen
hether in view of the similarities in the chemical and staining reactions the
uter membrane of the scorpion epicuticle may also be formed of paraffins.
t has been pointed out that the insect waxes are easily dissolved by chloro-
rm and carbon tetrachloride. Application of these organic solvents showed
o effect on the layer in question. Similarly other solvents such as methyl
thyl ketone, decalin, carbon disulphide, ether, and acetone were tried with no
ffect. However, with benzene and toluene this layer appeared to dissolve.
o test this observation, pieces of cuticle were treated for prolonged periods
ith toluene; sections were prepared and stained with Mallory. It was obvious
hat the outer membrane was lacking in such preparations, owing to the sol-
ent action of toluene. A similar result was obtained with material treated
ith benzene.

X-ray diffraction studies

To confirm and extend the observations reported above, special prepara-
ions of the scorpion cuticle were studied by X-ray diffraction methods.

Epicuticle prepared by treatment with chlorated nitric acid would conta both the inner basal layer and the outer thin membrane. To make sure th specimens used for X-ray examination contained these two layers, sma samples of such material were sectioned and stained to see if both the laye in question were present. Another set of such epicuticular layers was treat with toluene or benzene, which have a solvent action on the outer membra (see above). This treatment was prolonged till the outer layer had complete dissolved, as was shown by examination of stained sections of such materia In this way the inner basal layer of the epicuticle was separated and studi by the X-ray diffraction method. Fig. 2, C and D, shows the diffraction patte of the inner basal layer of the epicuticle. The prominent *d* spacings are 3·37 Å, 4·1 Å, and about 9·1 Å. This pattern does not agree with that arthropodin, which, as Fraenkel and Rudall (1947) observed, conforms to t β or extended configuration. The prominent *d* spacings observed in the di fraction pattern of arthropodin are at 4·5 Å and 9·8 Å, while the 3·4 Å appea to be very faint. A comparison of this with the one obtained for the protein the scorpion epicuticle shows that in the latter the reflections correspondi to the 4·5 Å and 9·8 Å are not evident, while the most prominent one 3·37 Å. In the above features the epicuticular protein of the scorpion diffe from other fibrous proteins of either the α or β configuration. It has alrea been pointed out that the chemical properties of this protein are peculiar some respects, for unlike other fibrous proteins, it survives such a viole treatment as is involved in the chitosan preparation, in which the cuticle heated for 4 to 6 hours in concentrated potash at 180° C.

The unique features in the X-ray diffraction pattern of the epicuticular pr tein of *Palamneus* make it difficult to relate it either to arthropodin characte istic of insect cuticle or to the keratin of vertebrates, although the presence sulphur linkages may suggest a relationship to the latter. Brown (195 pointed out that the mere presence of the disulphide linkages may not justi the assignment of a structural protein to vertebrate keratin unless it all shows a diffraction pattern identical to it. A comparison with the diffracti pattern of human hair, which is typically of the keratinous type, brings o marked differences. It appears likely that the hardened protein of the epic ticle of the scorpion is different from arthropodin or its derivative sclero and shows properties which suggest a relationship to the keratin of vertebrat with which, however, it does not agree in the X-ray diffraction pattern.

The preparations of the epicuticle containing both the inner basal layer a the outer membrane yield a diffraction pattern which in addition to the spacings characteristic of the protein component, shows well marked rin

---

FIG. 2. X-ray diffraction figures of the cuticle of *Palamneus swammerdami*. The X-ray be was parallel to the surface of the cuticle in B, perpendicular in all the others. The distan from the object to the film was 4 cm. in A–C, 5 cm. in D–F.

A and B, entire cuticle.

C and D, epicuticle without its outer membrane.

E and F, epicuticle with its outer membrane.

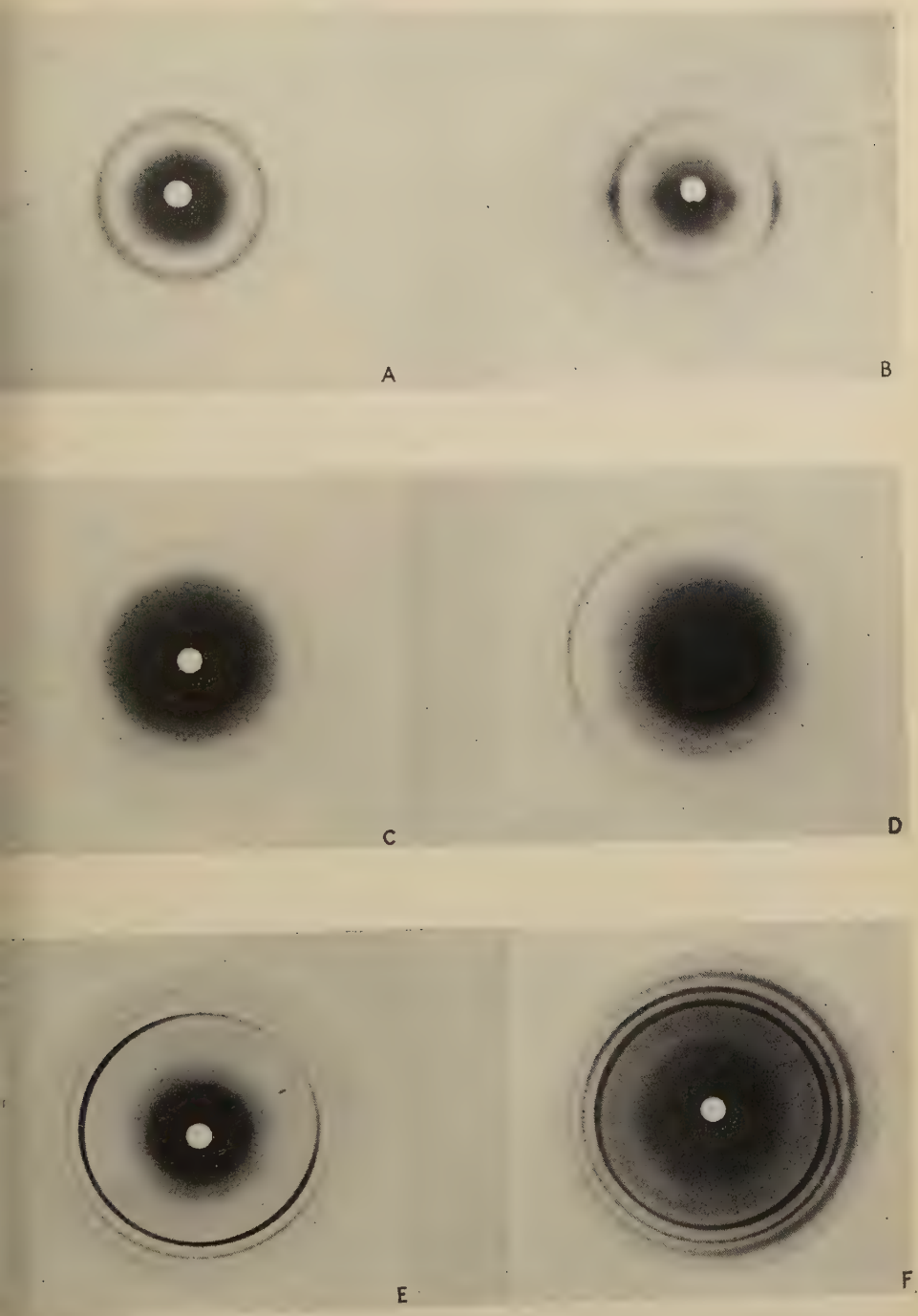


FIG. 2

G. KRISHNAN

rresponding to prominent *d* spacings 2·22 Å, 2·44 Å, 3·7 Å, and 4·14 Å (fig. E and F). These spacings show an identity with those obtained for long-ain paraffins (C_{29} or thereabouts) (Muller, 1928). This pattern is obviously ıe to the thin outer membrane of the epicuticle and affords evidence that this yer is formed of paraffins. This is in accord with the inference that may be ade from the chemical tests recorded in the foregoing pages. Bergmann 938) noted that in insects the outermost layer of the cuticle contains paraffins $_{27}$–C_{31} or thereabouts). Dennell and Malek (1953) using X-ray diffraction ethods noted the presence of straight-chain paraffin in the outer epicuticle the blowfly larva.

The X-ray diffraction pattern obtained for pieces of entire cuticle shows n identity with that of the insect cuticle. Fig. 2, A and B, shows the patterns hen the X-ray beam passed perpendicular and parallel to the surface of the ıticle respectively. A close agreement with the corresponding patterns otained for the stick-insect cuticle is obvious (see Fraenkel and Rudall, 947). As has already been pointed out (Fraenkel and Rudall, 1940), X-ray iffraction studies of the entire cuticle yield only the molecular configuration f the chitin component, on account of the fact that the reflections of the non-hitinous substances could not be distinguished from the much stronger chitin attern. The principal *d* spacings of the chitin of *Palamneus* agree very losely with those of insect chitin, i.e. 3·3 Å, 4·55 Å, 5·0 Å, 9·9 Å (fig. 2, A). rom the above evidence it may be inferred that the chitin of the scorpion ıticle agrees well with that of insects. It is of interest to point out that the uticle of *Limulus*, which has been considered an outstanding exception in the rthropoda and which resembles the cuticle of the scorpion in many respects see Krishnan, 1953), is identical with that of the crustacean *Cambarus* Richards, 1949). This suggests that the chitin component of the cuticle is imilar in all arthropods.

The outer layers of the epicuticle

According to the picture of the insect cuticle suggested by Dennell and Malek (1953), the polyphenol, wax, and cement layers (tectocuticle) lie external o the paraffin layer, which forms the peripheral zone of the basal lipoprotein ayer. In the scorpion there is no evidence of the presence of a separate olyphenol layer. The ferric chloride test is negative throughout the epicuticle nd the positive argentaffin reaction noted in the outlying region of the basal picuticle may not be indicative of polyphenols; it will be shown later in this aper that the reaction is due to the presence of purines. In *Rhodnius* and *Tenebrio* the secretion of polyphenols is evidenced by the appearance of rgentaffin droplets at the tips of the pore-canals in preparation for the harden-ng by phenolic tanning (Wigglesworth, 1947, 1948). In view of the absence f tanning in the epicuticle of the scorpion, it is unlikely that polyphenols are ecreted into it. Further, Dennell and Malek (1953) observed that in *Peri-laneta* the argentaffin material that is passed into the epicuticle before

tanning is only a protein and that a polyphenol layer does not form a co stituent layer of the epicuticle. In the scorpion not only is there no evider of polyphenols, but a cement layer (tectocuticle) does not occur eith Neither chemical tests nor mechanical contrivances involving celloidin fil or durofix revealed the presence of a layer corresponding to the cement lay in the insect cuticle.

Purines of the Epicuticle

The outer membrane and the outlying zone of the basal lipoprotein lay give a positive Fehling test on prolonged treatment. The region giving t reaction is also positive to the murexide test. Entire pieces of cuticle we treated with concentrated nitric acid and evaporated to dryness in a wa bath. On addition of dilute ammonium hydroxide the cuticle turned a de purple colour and with dilute sodium hydroxide the coloration was mo violet. The above colour reactions were more intense with the cuticle of t juvenile specimens. When, after the application of the murexide test, t cuticle was sectioned and stained with Mallory, the outermost membra which normally stains blue now took up a red colour. Not only the outerm membrane but a narrow outer zone of the basal layer in immediate cont with it also stained, while the rest of the epicuticle did not take up the sta The murexide test was applied to the frozen sections of the cuticle (Lis 1936) in an attempt to locate the purines indicated by the above test. Cuti in different growth-stages was used for the application of the test, and it w of interest to note that in the earlier stages of growth before the cuticle w fully hardened the murexide test was positive in the epicuticle, while in t fairly hardened cuticle the reaction appeared more intense in the exocutic Additional evidence of the occurrence of purines is given by the positive reacti with ammoniacal silver nitrate in the outer region of the epicuticle, althou after prolonged treatment the entire epicuticle takes up a light brown colo

Additional tests were performed to confirm the occurrence of purines a their localization in the cuticle. Although such tests lack specificity, confirm tory evidence was obtained by the use of Cowdry's modification of t Courmont-André method (see Glick, 1949). The outermost narrow zone the epicuticle as well as the exocuticle were coloured dark brown, indicati the presence of urates. However, in view of their diffusibility it is difficult define precisely the regions where the purines occur. But from an examinati of a large number of sections, it may be inferred that the occurrence of puri is related to the regions of the cuticle hardened by —S—S— bonding.

The application of St. Hilair's method for the detection of purines (s Lison, 1936) on sections did not yield successful results, but with ent pieces of the cuticle an unmistakable red coloration was obtained. The absen of a positive reaction in sections of the cuticle suggests that the copper sa of purines that could be precipitated for transformation into the red fer cyanide may be in too low a concentration to render a visual demonstrati possible.

Discussion

From the foregoing study it is apparent that the epicuticle of the scorpion ffers from the insect epicuticle in a number of structural and chemical atures, as for example the absence of cement and polyphenol layers, the on-occurrence of phenolic tanning, and the mode of hardening by —S—S— onding. Despite these differences a fundamental similarity between them ay still be noted. Dennell and Malek (1953) suggested that the basic pattern f the insect epicuticle is similar to that of the two-layered condition that ccurs in the larva of *Sarcophaga*. When compared with such an epicuticle the atures seen in the scorpion reveal a close similarity. Like the inner and outer picuticle of *Sarcophaga*, the basal layer and the outer membrane stain red nd blue respectively with Mallory. As in the insect epicuticle the inner layer ives evidence of lipoproteins, although the nature of the protein appears ifferent and it is not certain that the lipoidal constituent is a steroid, as stated y Dennell and Malek (1953). In insects the protein of the cuticle comprises water-soluble and water-insoluble fraction. Fraenkel and Rudall (1947) elieved that the water-soluble fraction, which forms a considerable propor- ion of the protein of the soft cuticle, is of a similar nature in all arthropods; hey therefore called it arthropodin. A feature of arthropodin is that it occurs aturally in the extended or β configuration, which is unusual in structural roteins (Rudall, 1947). But such an occurrence seems to be related to the ormation of mixed lattices with chitin, for arthropodin in the β configuration hows agreement with chitin in lattice dimensions. Arthropodin is charac- erized chemically by a high tyrosine and low glycine content, and a more or less complete absence of cystine or cysteine. The epicuticular protein of *Palamneus* is markedly different, not only in the absence of tyrosine and in he presence of a considerable amount of cystine, but also in its molecular onfiguration as revealed by its X-ray diffraction pattern, which does not correspond with that of arthropodin. In the possession of —S—S— linkages it appears to agree in some respects with keratin, but is not identical with it. The significance of such unique features is not obvious.

The presence of paraffins in the outer membrane of the epicuticle is revealed both by chemical tests and by X-ray diffraction methods. There can be no doubt that this membrane has a constitution similar to that of the epicuticle of *Sarcophaga*, with which it appears to be homologous. The absence of polyphenol, wax, and cement layers is, however, striking. But even in insects these layers are variable in their occurrence. Richards (1951) considered it an open question how far these layers are sufficiently distinct and separate in origin to make it possible to establish their homology even within the class Insecta. Similarly, Kramer and Wigglesworth (1950), in their study of the epicuticle of the cockroach, asked 'whether for some insects the laminar picture of the epicuticle may not be too schematic'. Further, Dennell and Malek (1953) suggested that the wax and cement layers may be variable in their occurrence, being present in some insects and not in others. A similar

variability in the occurrence of the cement layer may be inferred from the obse vation of Lees (1946, 1947) that in ticks, in which the epicuticle is identical wi that in insects, a cement layer is lacking in *Ixodes* although present in t allied *Ornithodorus*.

In the light of these observations, the epicuticle of the scorpion may said to conform to the basic type found in insects, the principal differenc being attributable to the occurrence of —S—S— bonding and the chemic constitution correlated with it. It is suggestive that the absence of polyphen and cement layers in the scorpion may be related to the non-occurrence phenolic tanning. For, if phenolic tanning does not take place in the epicutic polyphenols can hardly be expected to occur there. A cement layer, again, scarcely to be expected, for protein and polyphenols are involved in formati of cement. The other chemical peculiarities of the scorpion epicuticle may l explained similarly. For example, the occurrence of purines in the scorpi cuticle may be associated with the formation of —S—S— bonds. It has be pointed out by Huggins and others (1951) that proteins such as seru albumin and globulin are converted by urea into firm transparent gels in whi sulphydryl groups play an important part. An explanation of the role of ur is that in its presence a consecutive process occurs in which the free sulphydr group of one albumin molecule reacts with a disulphide group in a neigh bouring molecule to form an intermolecular disulphide bond. A similar ro has been ascribed to guanidine hydrochloride by Putnam and Neura (1945). In view of such observations it may be reasonable to infer that th purines noted to occur in the cuticle of the scorpion may play a part simil to that of urea and guanidine hydrochloride in the formation of disulphi bonds, resulting in the hardening of the epicuticle. Purines have been reporte to occur in the cuticle of spiders (Millot, 1926), but very little is known of th structural and chemical features of the spider epicuticle and its mode o hardening. If it should be constituted as in the scorpion and hardened b —S—S— bonds it would afford supporting evidence in favour of the abov assumption.

The differences between the epicuticle of the scorpion and that of tic (Lees, 1946, 1947), which resemble closely the condition seen in insects suc as *Rhodnius*, render it difficult to generalize on the epicuticular constitution o arachnids. What little is known of the epicuticle of other arachnids (Browning 1942; Lafon, 1943*a*, 1943*b*; Cloudsley-Thompson, 1950; Sewell, 1951 suggests that in them the epicuticle may not conform to a common pattern It appears probable that two distinct types based on the mode of hardenin may occur in this class, one approximating to the condition met with i insects, as exemplified by the ticks (Lees, 1946, 1947), and the other charac terized by the occurrence of —S—S— bonding as seen in the scorpion (Krish nan, 1953) and *Limulus* (Lafon, 1943*a*). It is questionable how far th differences between the two types can be explained as due solely to th occurrence or absence of —S—S— bonding. We do not know the significanc of such differences.

This work was carried out at the Zoological Research Laboratory, University Madras. I am indebted to Prof. G. N. Ramachandran and his student, Mr. artha, for taking the X-ray photographs and for the valuable help given me their interpretation. I am grateful to Prof. R. Dennell of the University of anchester for helpful suggestions in the course of this study. My thanks e due to Prof. C. P. Gnanamuthu for his interest and support. I am grateful the authorities of the University of Madras for the generous facilities forded me.

REFERENCES

ERGMANN, W., 1938. Ann. Ent. Soc. Amer., **31**, 315.
ROWN, C. H., 1950. Quart. J. micr. Sci., **91**, 331.
ROWNING, H. C., 1942. Proc. Roy. Soc. B, **131**, 65.
LOUDSLEY-THOMPSON, J. L., 1950. Nature, Lond., **165**, 692.
ENNELL, R., 1946. Proc. Roy. Soc. B, **133**, 348.
— 1947. Ibid., **134**, 485.
ENNELL, R., and MALEK, S. R. A., 1953. Nature, Lond., **171**, 298.
RAENKEL, G., and RUDALL, K. M., 1940. Proc. Roy. Soc. B, **129**, 1.
— 1947. Ibid., **134**, 111.
LICK, D., 1949. *Techniques of histo- and cyto-chemistry*. London (Interscience).
UGGINS, C., TAPLEY, D. F., and JENSEN, E. V., 1951. Nature, Lond., **167**, 592.
RAMER, S., and WIGGLESWORTH, V. B., 1950. Quart. J. micr. Sci., **91**, 63.
RISHNAN, G., 1951. Ibid., **92**, 333.
— 1953. Ibid., **94**, 11.
AFON, M., 1943*a*. Bull. Inst. Oceanogr. Monaco, No. 850.
— 1943*b*. Ann. Sci. nat. Zool., **5**, 113.
ÁNGNER, E., 1937. Zool. Jb. (Abt. Anat) **63**, 483.
EES, A. D., 1946. Parasitology, **37**, 1.
— 1947. J. exp. Biol., **23**, 379.
ISON, L., 1936. *Histochimie animale*. Paris (Gauthier-Villars).
ILLOT, J., 1926. Bull. Biol. Fr. Belg., Suppl., **8**, 1.
ULLER, A., 1928. Proc. Roy. Soc. A, **120**, 437.
UTNAM, F. W., and NEURATH, H., 1945. J. biol. Chem., **160**, 239.
ICHARDS, A. G., 1949. Science, **109**, 591.
— 1951. *The integument of Arthropods*. University of Minnesota Press.
UDALL, K. M., 1947. Biochem. Biophysica Acta, **1**, 549.
EWELL, M. T., 1951. Nature, Lond., **167**, 857.
IGGLESWORTH, V. B., 1947. Proc. Roy. Soc. B, **134**, 163.
— 1948. Quart. J. micr. Sci., **89**, 197.

A Study of the Osmium Techniques for the 'Golgi Apparatus'

By JOHN R. BAKER

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

With one plate (fig. 1)

Summary

The paper is concerned with the action of the osmium techniques for the 'Golgi pparatus' on the intestinal epithelial cell of the mouse. It is shown that, when the xative has acted, there are many separate lipide spheres in the 'Golgi' region. During ostosmication the spaces between these spheres are filled up with a black precipitate. his black precipitate does not represent the distribution of lipide material.

Introduction

WHEN tissues are treated by the usual osmium or silver techniques for the 'Golgi apparatus', a black or blackish deposit of osmium (probably n the form of hydroxide) or of silver is formed in a particular place (or places) n the cell. According to the view that is usually adopted, this black deposit ives us a representation of the distribution in life of a special lipoidal or pide-containing substance, the 'Golgi substance'. The unsaturated bonds of ne lipide are supposed to have reduced the osmium tetroxide or silver nitrate.

The purpose of the work described in the present paper has been to test this ypothesis of the significance of the black precipitate formed in osmium reparations.

Only one kind of cell has been carefully studied, namely, the absorptive cell f the intestinal epithelium of the mouse. (The Paneth cells of the crypts of Lieberkühn are briefly mentioned on p. 387.) The object has been to ascertain he distribution of lipidė material when the cell has been fixed by the standard Golgi' fixatives, and to compare this distribution with that of the black pre-ipitate formed during postosmication.

It has been proved, by easily confirmed experiments, that in this particular ell the black substance seen in ordinary 'Golgi' preparations does not repre-ent the distribution of lipide-containing material.

Methods

The work has been done on adult mice. Since fat-absorption prevents one rom getting a clear view of the cytoplasmic inclusions of the cell, mice were isually kept on a diet of bread and water for 24 hours and then with water nly for 4 hours, before being killed with chloroform. (I have never found that illing with chloroform affects the distribution of lipides in cells.) Instead, nice were sometimes kept overnight without food (but with water), and killed n the morning. It must be remarked, however, that when food is made con-

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 383-388, September 1954.]

tinuously available to mice, the stomach and first part of the small intestine a
often found to be empty, and good 'Golgi' preparations can then be made

The part of the intestine chosen for study was between 3 and 8 cm. from t
pylorus. Pieces about 1 cm. long were cut out as soon as the mouse was dea
and placed in the fixative. It was found convenient, as a general rule, to slit t
piece open longitudinally with fine scissors after the fixative had acted f
about 4 minutes. If the slit was made before fixation began, the piece tended
become twisted.

The investigation was carried out in two series of experiments.

In one series the standard 'Golgi' techniques were used. The two techniqu
chosen for special study were those of Weigl and of Kolatchev.

Weigl himself (1910) did not give very precise instructions. In the prese
investigation, the pieces of intestine were fixed for 4 hours in Mann's flu
(1894) and then washed overnight in running water. As a general rule,
to 14 days' postosmication in 2 per cent. aqueous osmium tetroxide soluti
at room-temperature (with changes of the fluid) gave the typical 'Gol
appearance. After being washed, the pieces of tissue were embedded
paraffin, sectioned at 3μ, and mounted unstained in balsam.

Kolatchev (1916) used various mitochondrial fixatives, washed thorough
and then postosmicated. He seems to have favoured Meves's fixative (1908), b
also sometimes used Champy's (1911). Those who have followed him ha
nearly always used either Champy's fluid or Nassonov's (1923) modification
it. I have generally used Champy's fluid and have adhered carefully to Kol
chev's directions. The duration of fixation is 24 hours.

It may be remarked that there is no advantage in using Nassonov's modific
tion of Champy's fluid, though for some unexplained reason nearly everyo
who has used the Kolatchev technique in recent years has substituted Nass
nov's fixative for Champy's. The latter's fluid consists, as is well known, o
mixture of 3 per cent. potassium dichromate, 1 per cent. chromic acid, a
2 per cent. osmium tetroxide in the proportion of 7:7:4. Nassonov used t
proportion of 4:4:2. He does not say why he changed the proportion slight
nor even that he did so. He simply calls the 4:4:2 mixture 'Champy's flui
He recommended the addition of 2 or 3 drops of 0·1 per cent. pyrogallol
10 c.c. of the mixture. The addition of pyrogallol, a reducer, to about 2,0
times its weight of three strong oxidizers, does not commend itself to reas
and indeed I have found no difference between the action of Champy's flu
and that of Nassonov's modification.

The fixative must be very thoroughly washed out in running water. For t
intestinal epithelium, a period of 9 days' postosmication in 2 per cent. osmiu
tetroxide at 34° C. is about right, though sometimes one will get better resu
with shorter or longer times. It is best to renew the solution several times du
ing the 9 days. After thorough washing in running water, the tissue is embedd
in paraffin, sectioned at 3μ, and mounted unstained in balsam.

In the second series of experiments, often performed with material from t
same mice, the same fixatives were used for the same periods, and the piec

ere then washed thoroughly in running tap-water. They were not post-smicated, however, but instead were embedded in gelatine and sectioned at 5μ or 8μ on the freezing microtome.

The sections of Mann material were treated with a 1 per cent. solution of odine in 2 per cent. aqueous potassium iodide solution for a few minutes, to et rid of the mercury precipitate, and then with 5 per cent. sodium thiosul-hate solution for about a minute; they were then thoroughly washed in water.

The sections of Mann, Champy, and Nassonov material were coloured with udan black according to my usual method (1949). Staining of chromatin with armalum was generally omitted, however, for it does not work after Champy r Nassonov fixation, and it is not necessary after Mann. A trial of many ifferent mounting media confirmed my belief that Farrants's is as good as ny for this purpose. Preparations seem to improve gradually by becoming nore transparent over a period of days or weeks.

It sometimes happens that the osmium in the *fixative* makes parts of the cell grey. This could be misleading, for the darkness might be attributed in error o the Sudan black. In cases in which it was thought that error might result from his cause, sections were bleached with 1 per cent. aqueous sodium iodate olution or saturated aqueous potassium persulphate solution before being coloured with Sudan black.

Results

The crucial point of the investigation was the comparison of the results btained by postosmication with those obtained by the action of Sudan black. f the usual view of the nature of the 'Golgi apparatus' were correct, the form f the apparatus in osmium preparations would be the same as that seen after he use of Sudan black, since the latter agent reveals the sites of lipides.

It will be convenient to describe the Sudan black preparations first. Whether the fixative was that of Mann (fig. 1, A), Champy (fig. 1, C and E), or Nassonov, the appearance given is the same. A large number of spherical or subspherical bodies, coloured with Sudan black, form a group in the cytoplasm near the 'apical' end of the oval nucleus—that is to say, near the end that is directed towards the free border of the cell. I call such lipoidal bodies *lipochondria*. A few of them sometimes occur along the sides of the nucleus, but the majority occur in the group near its apical pole. The extension or non-extension of the lipochondria (and also of the osmium precipitate in 'Golgi' preparations) beside the nucleus appears to depend on the shape of these very elastic cells at the moment of fixation. When the cell is very long and thin, they are generally restricted to a group near the apical end of the nucleus.

The lipochondria are separate from one another. They vary considerably in size in a single cell, but the diameter is commonly between $0{\cdot}4\ \mu$ and $1{\cdot}4\ \mu$. They are smallest and least strongly coloured towards the base of the villus, and largest and darkest towards its tip. They seem to be homogeneous in these preparations, though when the intestine is fixed in formaldehyde solution and postchromed they often appear to be vacuolated (see Baker, 1949).

If a piece of intestine is fixed in formaldehyde-saline and sectioned on th freezing microtome, and the sections treated with Sudan IV, the lipochondr are not coloured. It is clear, therefore, that they are not simply globules of tr glyceride. Their chemical composition is being investigated by Dr. W. G. I Casselman at Oxford.

In standard 'Golgi' preparations made with the same fixatives, but wit postosmication instead of treatment with Sudan black, the appearance different. There is considerable 'capriciousness' in these techniques, especiall Kolatchev's. However hard one may try to reproduce exactly the condition that gave a successful impregnation, one may fail at the second attempt, onl to achieve a good result again on some later occasion. In general, it is mo usual to obtain good impregnation at the bases of the villi than at their tip The cells of the crypts of Lieberkühn are regularly impregnated.

The impregnated region in the absorptive cells of the epithelium of the vil is the same as that in which the lipochondria were seen in the Sudan blac preparations: that is to say, it lies in the cytoplasm near the apical pole of th nucleus, and sometimes extends alongside the nucleus.

The structure of the impregnated zone in 'Golgi' preparations is very differ ent from that seen after Sudan black. In any one cell the osmium usually ap pears in the form of a single black mass of irregular shape (see fig. 1, D, F, an H). Sometimes it contains unimpregnated spheres or 'vacuoles'; some of thes are indicated by arrows in the photomicrographs. Similar vacuoles are some times seen in Aoyama (silver) preparations (fig. 1, G).

These facts prove that the black material seen in osmium preparations doe not represent the lipide that was present in the cell at the end of fixation. O the contrary, it represents either the whole region of the cell in which the lipo chondria were situated, or else the parts of the cytoplasm *between and aroun* the lipochondria. It seems likely that the 'vacuoles', when present, represe the lipochondria, and that the osmium is commonly deposited on their su faces and between them. Whether the osmium is deposited also *in* the lipo chondria, in those cases in which vacuoles are not seen, is uncertain.

It is very enlightening to examine Weigl preparations that have been post osmicated for a shorter period (about 8 days) than that which gives the typic 'Golgi' picture. In such preparations (fig. 1, B), only the lipochondria are im pregnated with osmium. One cannot say for certain whether the osmium ha been deposited on their surfaces only, or in their substance, but none is de posited in such a way as to fill up the spaces between them. The appearance very similar to that given by Sudan black, though fewer lipochondria are seen because the section is much thinner. The facts strongly suggest that furthe osmication simply fills up the spaces in between.

FIG. 1 (plate). All the photomicrographs represent sections through the intestinal epith lium of the mouse. The free border is uppermost in each case. The letters A, B, C, &c., a given for ease of reference in the text. The arrows point towards 'vacuoles' in classical 'Golg preparations.

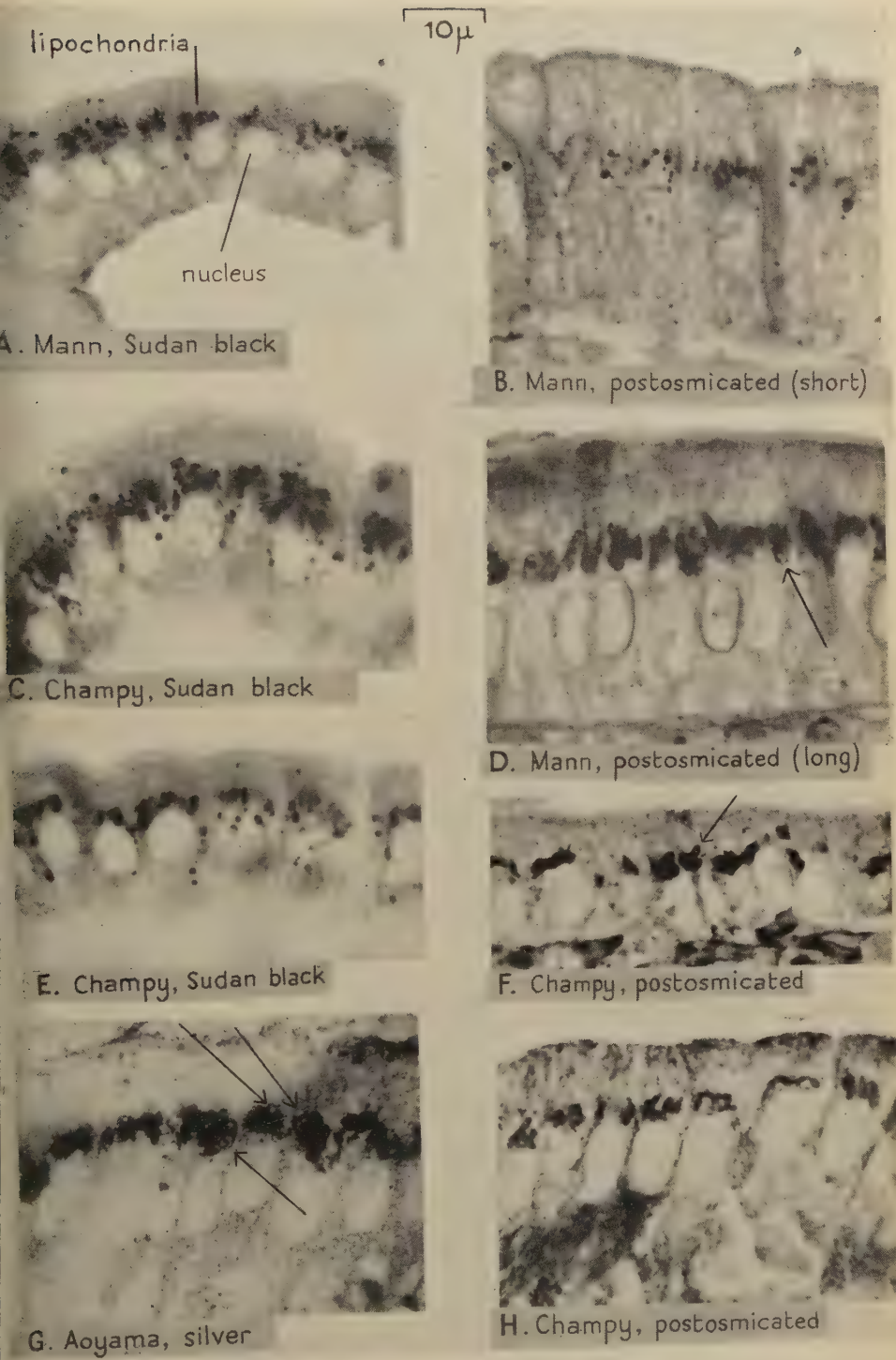


FIG. 1

J. R. BAKER

Thomas (1948) used the Weigl technique in his study of the sympathetic nerve-cell of the mouse. He postosmicated for different periods from 12 hours upwards. He found that when the period of postosmication was short, only the spheroids' that exist in the cytoplasm of nerve-cells during life were impregnated. When the period of postosmication was increased, osmium began to be deposited partly on the mitochondria, and partly also at random (that is to say, in such a way as not to represent anything that was present in the living cell). The findings recorded in the present paper confirm Thomas's discovery that at first only the spheroids (lipochondria) are impregnated by Weigl's method.

Mann and Champy fixation followed by the colouring of gelatine sections with Sudan black are useful techniques for showing lipide droplets in a wide variety of different cells. I have tried many different fixatives, simple and mixed, including many new mixtures. I have not found any fixative that is better than Mann's or Champy's for this purpose, though Meves's is also very good.

Discussion

It follows inevitably from the easily repeatable experiments described in the present paper that the greater part of the osmium seen in 'Golgi' preparations of this particular cell does not represent the site of lipide that was present there at the end of fixation. Two possibilities present themselves:

(1) The osmium was deposited on the surfaces of (or perhaps sometimes in the substance of) the lipochondria, and further particles of osmium were added one to another, like silver in a photographic plate during development, until the intervening spaces were filled. If so, the standard 'Golgi' techniques give a very misleading impression of what was present at the end of fixation.

(2) There exists a reducing substance, *not a lipide*, between and around the lipochondria. Of this possibility it can only be said that we have as yet no clear evidence of the existence, far less of the chemical composition, of the hypothetical substance. Still, its existence remains a possibility.

The large spherical granules of the Paneth cells in the crypts are not coloured by Sudan black, nor are the spaces between them. In 'Golgi' preparations a three-dimensional network of osmium extends between the granules. It is obvious that in this case also the osmium does not represent the site of lipide, and the two possibilities just mentioned present themselves.

I have described elsewhere (1949) how the villi may be removed and placed in saline solution for microscopical examination in a fresh condition. Spheroids can be seen in the cytoplasm near the apical pole of the nucleus. It can be argued that these may represent lipide droplets that have separated from lipoprotein complexes as a result of the treatment of the villi, and that they would not have been seen in this form if the cell could have been examined while the villus remained in connexion with its blood supply. Similarly, the lipochondria seen in Sudan black preparations may conceivably represent a result of separation of lipide from lipoprotein, caused by the action of the fixative. This possibility does not alter the inevitable conclusion that must be drawn from the

facts recorded in this paper. The lipochondria are certainly present at the en of 'Golgi' fixation, whether one is going to postosmicate or to colour with Suda black—and there is no lipide between them.

Prof. A. C. Hardy, F.R.S., has kindly continued to support my studies o the lipide constituents of cells. I acknowledge a great deal of skilful practica assistance from Miss B. M. Jordan. Dr. W. G. B. Casselman has given me th benefit of his criticism of the manuscript of this paper.

REFERENCES

Baker, J. R., 1949. Quart J. micr. Sci., **90**, 293.
Champy, C., 1911. Arch. d'Anat. micr., **13**, 55.
Kolatchev, A., 1916. Arch. russes d'Anat. d'Hist. d'Emb., **1**, 383.
Mann, G., 1894. Zeit. wiss. Mikr., **11**, 479.
Meves, F., 1908. Arch. mikr. Anat., **72**, 816.
Nassonov, D., 1923. Ibid., **97**, 136.
Thomas, O. L., 1948. Quart. J. micr. Sci., **89**, 33.
Weigl, R., 1910. Bull. internat. Acad. Sci. Cracovie B (no vol. number), 691.

The Value of 'Spreading Factors' in the Demonstration of Tissue Neural Elements

By G. WEDDELL AND W. PALLIE

(*From the Department of Human Anatomy, University of Oxford*)

With three plates (figs. 1, 2, and 3)

Summary

1. A description is given of the value of 'spreading factor' (hyaluronidase) used in conjunction with various neurohistological methods for the display of tissue neural elements.

2. It has been shown that after its use nerve fibres and their terminals in a variety of tissues, including the cornea, closely resemble those seen in fresh specimens of cornea under phase-contrast conditions.

3. The specimens prepared after treatment with hyaluronidase are almost free from artifacts. The staining or impregnation process can be controlled with certainty and precision to produce evenly stained specimens in the tissues we have used.

4. It is suggested that some tissues may need special treatment to obtain optimal results.

5. The use of 'spreading factor' makes it possible to determine the cause of the artifacts commonly observed after employing standard neurohistological techniques.

6. The rationale of using 'spreading factor' in conjunction with neurohistological techniques is discussed.

Introduction

METHODS for displaying tissue neural elements suffer from defects of which two are outstanding from the point of view of functional interpretation. (1) The selective staining or impregnation of nerve fibres and their terminals is never completely uniform. Thus, contrast is lost and it becomes impossible to distinguish fine detail, a circumstance which has led some observers to conclude erroneously that no nerve fibres are present in particular positions in certain tissues. (2) The majority of neurohistological methods distort and some even destroy a proportion of the tissue elements. This makes it difficult either to determine the exact relationship of the different tissue elements to one another or to distinguish artifacts introduced by the method from structures present in the living state.

Since some distortion is inevitable in fixed tissues, the most reliable method of assessing the detailed anatomy of the finest tissue elements is to examine them in the living state. In transparent tissue such as the cornea, the anatomy of the neural elements can be assessed by examining fresh slices under phase-contrast conditions (Weddell and Zander, 1951). The nearest approach to this in less transparent tissues is to stain them with methylene blue in the living animal and to examine fresh preparations before the dye 'fades' (Ohmori, 1924). This method is notoriously capricious and, despite recent improvements in technique (Weddell, 1941; Feindel, Allison, and Weddell, 1948), it

[Quarterly Journal of Microscopical Science, Vol. 95, part 3, pp. 389-397, September 1954.]

is still not possible to control the concentration of the dye reaching a given tissue within the fine limits necessary for selective staining which will not distort the neural elements or give rise to artifact-formation. Attempts to 'fix' the dye and make the tissue available for microscopical examination as a relatively permanent preparation result in further distortion of the neural elements and the introduction of still more artifacts (Weddell and Zander, 1950).

Other methods of staining tissue neural elements (such as silver, gold, osmium tetroxide, iron haematoxylin, &c.) involve the submission of the tissue to an even longer and more complicated series of processes than vital methylene blue staining, all of which are liable to produce artifacts.

In an effort to overcome the defects of patchy staining, distortion of nerve fibres, and the introduction of artifacts during the process of preparing tissues for microscopical examination, we have exposed them to 'spreading factors' before subjecting them to specific neurohistological techniques. It is the purpose of this paper to give a general account of the results which we have obtained and to discuss the value of such enzymes and the rationale of their use for neurohistological purposes.

Material

Our observations were made chiefly on skin obtained from living animal and human subjects at operation, but we were also successful in obtaining good preparations from the cornea, mesentery, stomach, intestine, uterus, kidney, and suprarenal of the rabbit.

Method

A 1 c.c. ampoule of 'Hyalase' (Benger) equivalent to 1 mg. of hyaluronidase was diluted with 5 c.c. of normal saline and injected into the tissue (in the case of skin, both intra- and subcutaneously), the usual precautions with regard to strict cleanliness being observed. Not less than 20 minutes later, the tissue was carefully removed and fixed in 15 per cent. formalin in preparation for impregnating with silver according to the techniques of Weddell and Zander (1950), or of Bielschowsky-Gros, or for staining for myelin by Weil's method. In the case of methylene blue staining a solution of 0·02 per cent. or 0·04 per cent. methylene blue (Merk) in normal saline was infiltrated into the area treated with hyaluronidase until it was stained a uniform blue colour. The treated tissue was then massaged at about 5-minute intervals over a period of 20 minutes, after which it was removed and treated in the manner described by Weddell (1941).

Observations

Methylene blue staining by local injection

It is difficult to compare histological techniques of the kind in question objectively, for they are all capable of yielding an almost perfect result in a

nited region and, on rare occasions, over a wider area. It is, however, possible state that on 40 successive occasions we have obtained almost uniformly ccessful methylene blue staining of the cutaneous nerve fibres and their rminals in skin from the dorsum of the rabbit ear after the use of hyaluro-dase; whereas our notes show that in only 20 instances out of 40 were the sults uniform over even small areas of skin in preparations stained with ethylene blue, when hyaluronidase was not used. Moreover, in none of these ntreated cases were all the nerve fibres and their terminals perfectly dis-layed throughout the uniformly stained areas.

We do not claim that the use of hyaluronidase in the manner described by us ill make it possible for the axis cylinders of the nerve fibres to be stained niformly, specifically, and without distortion in all tissues and on all occasions. Ve have, however, been able to obtain consistently good results in all the ssues which we have examined and our standard for comparison has been ne appearance of the axis cylinders of nerve fibres teased apart from a nerve runk in normal saline and the appearance of fine axons in the fresh cornea nder phase-contrast conditions (Weddell and Zander, 1951).

One striking advantage of the method is the minimal pressure required to nject methylene blue into the tissue. Thus, with practice, it is possible to tain axons throughout the skin of the dorsum of the rabbit ear through a ingle puncture by subcutaneous infiltration of the dye solution. To stain erve terminals in the epidermis satisfactorily, however, it is necessary to nject the dye intradermally also. Various other modifications have to be nade, depending upon the tissue which is to be stained. In the case of the abbit uterus, for instance, the dye had to be injected intravascularly after the terus had been infiltrated with hyaluronidase, to obtain the best results Pallie, Corner, Weddell, 1953).

The effect of injecting hyaluronidase before the dye is twofold. Not only are he nerve fibres stained more uniformly over a wider area but they hardly ppear to be distorted at all. The finest nerve fibres are beaded, but the size of he beads is less than in preparations made without hyaluronidase and they are lways connected to one another by threads of axoplasm. If the concentration of methylene blue is increased, the size of the beads increases, and conversely. Low concentrations of dye may sometimes display fine nerve fibres which are virtually smooth in outline, but they are then difficult to see.

Fine nerve fibres ending in relation to hair follicles and those found within cellular capsules are particularly well seen, but we have found that such terminals, in whatever region of the body they are found, are best displayed if the strength of the dye is increased to 0·04 per cent., although this inevitably leads to some distortion (i.e. swelling and irregularity) of the axons serving them and to some generalized tissue staining.

These observations, together with more detailed findings, are best particular-ized by reference to a series of photomicrographs. Fig. 1, F is from a preparation of the skin of the rabbit's ear, stained with methylene blue. A single optical plane through a small nerve-bundle is shown. The axis cylinders are specifically

stained. They are smooth in outline and of uniform diameter. Nodes Ranvier can be seen in relation to each myelinated axon. Fig. 1, A is from similar preparation but at lower magnification and shows the manner in whic a nerve-bundle similar to that seen in the figure gives rise to a 'cutaneou plexus' of axons. In this preparation the concentration of methylene blue w 0·04 per cent., which has resulted in slight generalized tissue staining, t gether with staining of the nuclei of a number of cells in the connective tissu as well as those of the sebaceous glands. In addition, some of the finer, mo superficial, axis cylinders are not specifically stained but are seen to be en veloped by Schwann cells. Fig. 1, G is from a similar preparation and is in cluded to illustrate the distortionless, specific, and uniform staining of a isolated myelinated axis cylinder. Preparations of this kind provide an oppo tunity for measuring the lengths of axon between adjacent nodes of Ranvie

Fig. 1, C is from a similar preparation and again demonstrates the value hyaluronidase in obtaining undistorted preparations. The bundles of ax cylinders are displayed specifically and without distortion. The axons rema quite independent of one another and do not break up into neurofibrilla strands nor do they enter into a reticular formation. Fig. 2, A shows ho clearly it is possible to display fine axoplasmic filaments ending in relation the wall of a small artery. These filaments are derived from stem axons by process of arborization and each terminates freely and extracellularly. Th filaments do not fuse with one another to form protoplasmically continuou nets. This latter point is more convincingly demonstrated in Fig. 2, F, in whic axoplasmic filaments can be seen terminating freely in relation to a sma arteriole. Fig. 2, D shows fine axoplasmic filaments arising from stem fibre and ending in relation to a hair follicle in skin from the rabbit ear stained b methylene blue. It is very difficult to display the fine nerve terminals in th situation without prior treatment with hyaluronidase unless an excessivel high concentration of dye (0·05 per cent.) is used, and this leads to gross di tortion of the stem axons. High concentrations (0·04 per cent.) of dye can b used after treatment with hyaluronidase, for the fluid spreads so widely an evenly that the relative concentration of the dye is never excessive and thu distortion is minimal. Details of the innervation of hairs will be given in a sub sequent paper but our illustration shows the essential characteristic of nerv termination in the skin—stem axons giving rise to arborizations of separat axoplasmic filaments.

Fig. 1 (plate). A is from a specimen of skin from the rabbit's ear, stained with methyle blue (0·04 per cent.).

B is from a silver-impregnated specimen of skin from the rabbit's ear.

C is from a specimen of skin from the rabbit's ear, stained with methylene blue.

D is from a specimen of skin from the rabbit's ear stained by Weil's method.

E is from a silver-impregnated specimen of skin from the rabbit's ear.

F is from a specimen of skin from the rabbit's ear, stained with methylene blue.

G is from a specimen of skin from the rabbit's ear, stained with methylene blue.

H is from a silver-impregnated specimen (Romane's method) of nerve trunk from t rabbit's ear.

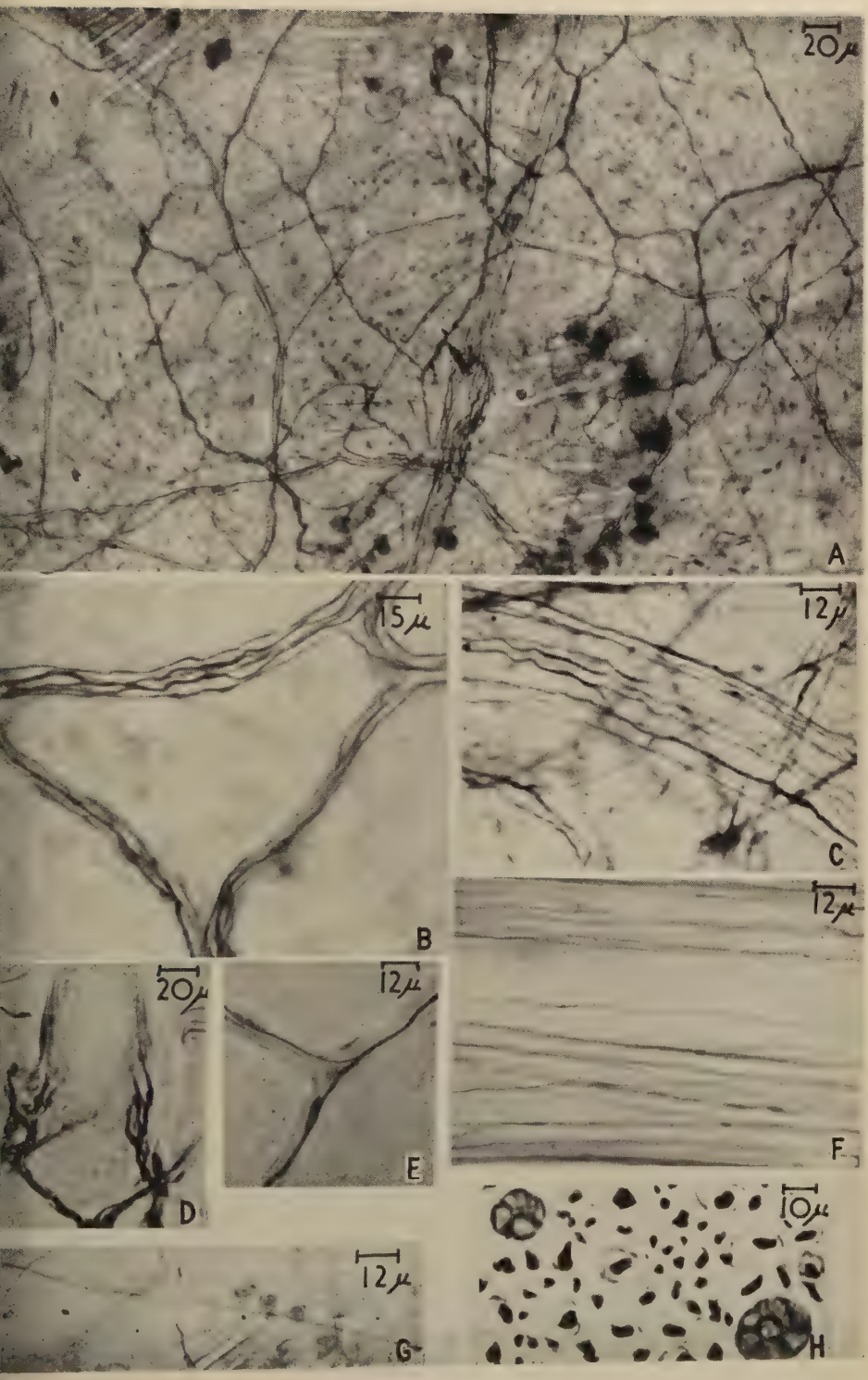


FIG. 1

G. WEDDELL and W. PALLIE

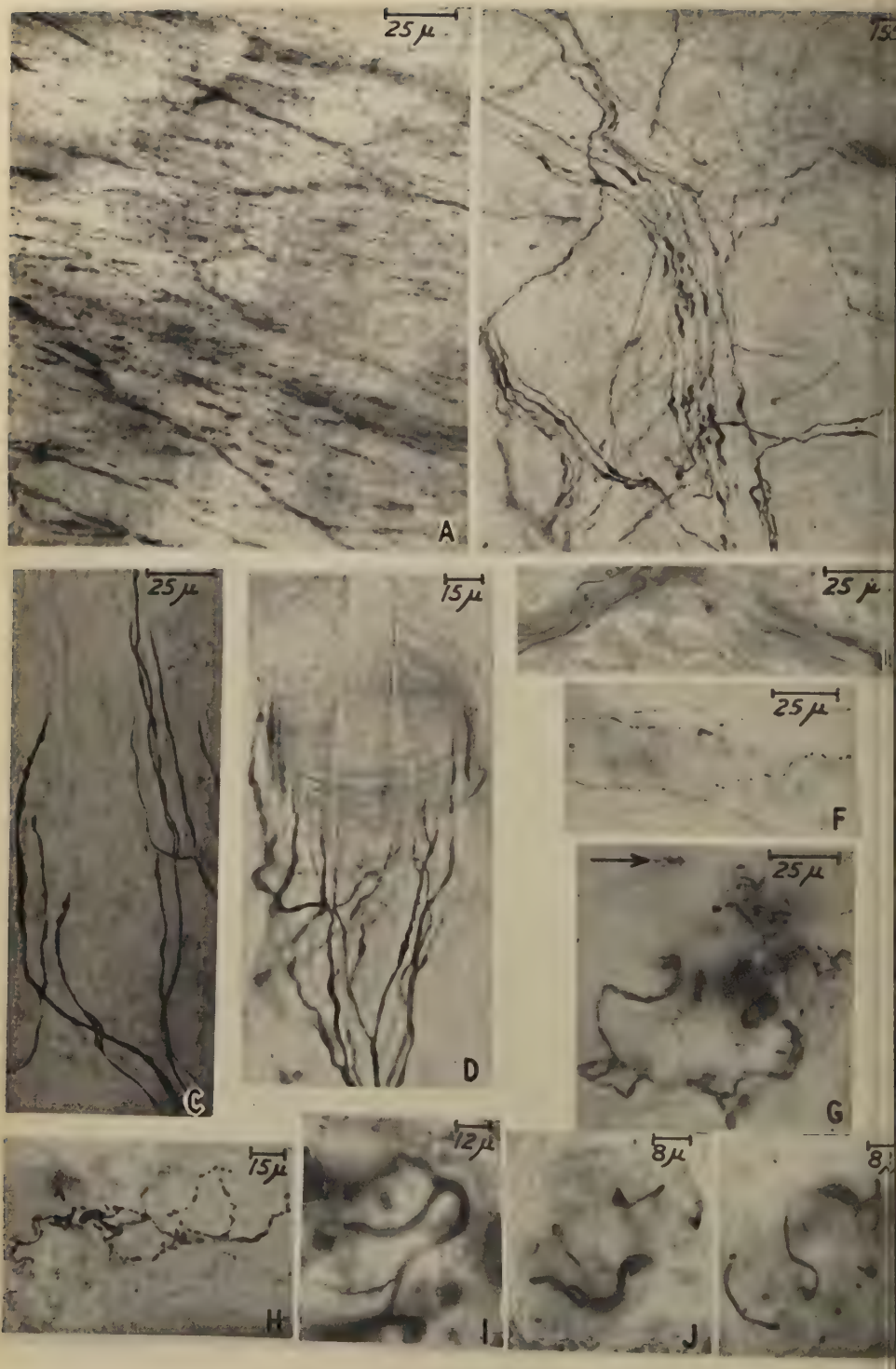


FIG. 2

G. WEDDELL and W. PALLIE

Fig. 2, G shows an encapsulated ending stained with methylene blue, from e pad of a human toe. It shows a myelinated axon giving rise to a series of e axoplasmic filaments which terminate freely and extracellularly, often in all swellings. These lie among the cells of the capsule which can only be en under phase-contrast conditions, for they are not stained with methylene ue in preparations of this kind. Fine terminals ending freely in between the lls of the stratum germinativum in the neighbouring epidermis can also be en; they are indicated by an arrow.

Fig. 2, H shows stem fibres giving rise to fine-beaded freely-ending naked oplasmic filaments in the epidermis of a rabbit tongue stained with methy- ne blue. The separateness and extracellular position of the terminals is early shown by the use of hyaluronidase, for the tissue can be fixed without stortion and the terminals do not collapse upon one another to simulate a otoplasmically continuous peripheral nerve net.

Fig. 2, B shows bundles of fine nerve fibres in the adventitia of the supra- nal gland of the rabbit. A number of fine filaments can be seen passing to e cortex where they ramify among the gland cells.

Fig 3, A–C are from rabbit stomach stained by local injection of methylene ue after treatment with hyaluronidase. The absence of shrinkage and distor- on of the fine nerve fibres and ganglion cells is striking. It is possible to llow individual axons in nerve bundles, where they always remain separate, wards their termination in the muscle coat or mucous membrane. The rmination always takes the form of an arborization into fine axoplasmic laments which end freely either in relation to a nerve ganglion cell or to one f the tissues of the stomach wall. We did not see any nerve fibres giving rise o a series of anastomosing fibrillae. Fig. 3, F is from a preparation of rabbit terus stained with methylene blue. It shows stem axons giving rise to fine aked axoplasmic filaments ending freely in relation to the uterine myome- ium. The nerve supply of the rabbit uterus has been the subject of a separate mmunication (Pallie, Corner, and Weddell, 1953).

ilver impregnation.

We have found that it is possible to obtain pictures comparable with those een after methylene blue staining if certain precautions are observed. The ssue should remain in fixative for at least 24 hours but not longer than 3–4

FIG. 2 (plate). A is from a specimen of skin from the rabbit's ear, stained with methylene lue.
B is from a specimen of suprarenal gland from the rabbit, stained with methylene blue.
C is from a silver-impregnated specimen of skin from the lip of the monkey.
D is from a specimen of skin from the rabbit's ear, stained with methylene blue.
E is from a silver-impregnated specimen of skin from the finger pad of the monkey.
F is from a specimen of the skin of the rabbit's ear, stained with methylene blue.
G is from a methylene blue stain specimen of skin from the pad of a human toe, stained ith methylene blue.
H is from a specimen of mucous membrane from the rabbit's tongue, stained with methylene lue.
I, J, and K are from silver-impregnated specimens of skin from the finger pad of the monkey.

days, and to obtain uniform impregnation it is essential that the sections sho be of uniform thickness. To impregnate the axon specifically it is necessary control the period during which the section remains in the ammoniated sil bath: with practice, this is not difficult. Careful control during this stage a makes it possible to demonstrate the relationship of axis cylinders to Schwann elements surrounding them. In this section, too, we shall attempt particularize our observations by reference to a series of photomicrograp but the results are less dramatic than in the case of methylene blue staini for sections as opposed to whole preparations give flat, strictly two-dime sional, pictures. It is also necessary to impregnate the surrounding tissue some extent, for otherwise the pictures are merely of fine black lines and t means that the finest axoplasmic filaments often appear as insignificant thre and do not stand out from the background.

Fig. 1, B is from a silver preparation of the skin of the rabbit's ear. It sho axis cylinders of the cutaneous nerve plexus specifically impregnated. Fig. 1 is from a similar preparation. In this case, however, the specimen was allow to remain slightly longer in the ammoniated silver bath and this has result in the Schwann cell sheath being clearly outlined. Fig. 2, C is from a silv preparation of skin from the lip of a Rhesus monkey. It shows stem fib giving rise to fine axoplasmic filaments ending in relation to a large hair follic The filaments are extremely fine and hence they do not appear so dark on t print as we would have liked. Moreover, it is difficult to display long lengths nerve in the single plane offered by a thin section. It will be noticed that this specimen the stem nerve fibres are slightly distorted. This is due to som what prolonged immersion in the ammoniated silver bath. As in the case methylene blue staining, the display of fine axoplasmic filaments in this po tion requires treatment which is rigorous enough to damage stem axons t small degree.

Fig. 2, E shows a stem axon giving rise to a leash of fine axoplasmic filamen coursing in close relationship to a capillary in the dermis of monkey skin. T filaments are fine and smooth and remain separate throughout their cour Fig, 2, I–K show encapsulated nerve endings from the finger pad of a monke In both pictures stem axons can be seen as well as the fine axoplasmic filamen to which they give rise. The filaments lie in between the cells of the capsu wall and end freely.

Fig, 3, D shows stem fibres giving rise to fine axoplasmic filaments endi on the cell bodies of nerve ganglion cells in the wall of the rabbit stomac The number of filaments is large but they are all separate and end freely in extracellular position. Fig. 3, E shows fine axoplasmic filaments which termi ate among the cells of the stomach mucous membrane of the rabbit. T filaments are derived from stem axons and end freely and extracellularly.

FIG. 3 (plate). A, B, and C are from specimens of rabbit's stomach, stained with methyle blue.

D and E are from silver-impregnated specimens of rabbit's stomach.

F is from a specimen of rabbit's uterus, stained with methylene blue.

FIG. 3

G. WEDDELL and W. PALLIE

The value of 'spreading factor' in relation to a more routine neurohistological procedure is illustrated in Fig. 1, H. This is part of a nerve trunk from dorsum of the rabbit ear which had been treated with hyaluronidase before moval and impregnation according to the method of Romanes (1950). The sence of gross distortion is particularly well illustrated in the appearance of capillaries.

yelin impregnation

Fig. 1, D shows myelinated axons in close relation to hair follicles in the bbit ear. The specimen was prepared by Weil's method and shows how the yelin sheath tapers towards its termination. There is comparatively little stortion, although impregnation was somewhat prolonged as can be seen by e faint but general nuclear staining.

Discussion

Duran-Reynals first demonstrated that the introduction of testicular extract to rabbit skin facilitated the spread of vaccinia virus in 1929 and it was soon scovered that the clinical use of this substance made it possible to introduce bcutaneously large quantities of fluids which diffuse readily without causing ain or damage.

We first employed hyaluronidase to promote the spread of methylene blue the skin of the rabbit's ear, as did McGregor (1953). We hoped that it would nable us to obtain more uniform staining of axis cylinders. Further acquaintnce with hyaluronidase, however, showed that not only does it assist the preading of methylene blue and the penetration of nerve-impregnating subtances, but it also alters the properties of the skin and other tissues to such an xtent that when they are fixed in either alcohol or formalin the axis cylinders re much less distorted by the fixative than usual. We also found that the esults of Weil's method were improved by preparatory treatment with spreading factor'.

The mechanism underlying the phenomenon of spread is complex but some f the factors are now known. The matrix of connective tissue is highly viscous nd consists of a complex of proteins and mucopolysaccharides. Its viscosity is lue in particular to hyaluronic acids, and the hyaluronidase enzymes, which re active principles in the testicular extract mentioned above, cause a chemical eaction which involves the depolymerization and hydrolysis of the hyaluronic cids, thereby greatly reducing the viscosity.

Since mucopolysaccharides are demonstrable histochemically in bloodessels in the dermis (more particularly in the region of the papillae) and round hair follicles, the introduction of hyaluronidase into the skin might have been expected to lower the viscosity in these regions and to facilitate the permeation of fluid throughout the skin generally. That this is the case we have demonstrated by the results obtained after the injection of methylene blue. Since the spaces between connective tissue fibrils and other structures are

rendered fluid, nerve fibres will be more freely and evenly exposed to stain or impregnating agents than is usually the case and thus distortion and a facts due to the physical process of the injection of methylene blue will virtually eliminated and, in addition, the concentration of the dye can be m accurately adjusted to obtain specificity of action without distortion, and staining process itself is more even.

Further, the use of 'spreading factors' assists the process of rapid and ev tissue fixation and thus eliminates artifacts which are known to result from slow penetration of fixatives. Similarly, the various agents used to stain impregnate nerve fibres after fixation penetrate more rapidly and evenly a the results obtained are thus far more uniform.

Finally, it has been shown in attempts made to wash out mucin from tissu that a viscous connective tissue matrix can, when subjected to tension, cau distortion and displacement of collagen fibres and presumably any ner fibres that may be passing among them. Thus artifacts resulting fr mechanical handling of tissues are also eliminated if the connective tiss matrix is rendered less viscous.

We do not claim to have exhausted the possibilities of this technique for o observations have so far been limited to certain specified regions of the sk Also, the effect of 'spreading agents' is known to be modified by age and the presence or absence of certain hormones, oestrogens tending to increase and corpus luteum hormones to decrease it. There are, in addition to hyalur nidase, lytic agents such as collagenase which might prove of great value specific instances. It is, however, clear from our observations that the use hyaluronidase as a 'spreading factor', when used in conjunction with each the three most widely used neurohistological methods (i.e. methylene bl staining, myelin and silver impregnation), gives sections in which the tiss elements are more consistently and uniformly stained and in which the ner fibres stand out so clearly that they can be traced to their ultimate point termination; further, they are relatively free from artifacts. This result is d to its property of allowing rapid and even penetration of fluids throughou tissues and its ability to render them relatively more homogeneous in the behaviour towards various reagents. The observations of Irving and Tom (1954), which have appeared since this paper went to press, suggest that the case of silver impregnation, hyaluronidase also renders reticular a collagen fibres non-argyrophil.

There is one other point worth mentioning, which concerns skin in partic lar. Khanolkar (1951) has recently clarified some essential features of lepro and has shown that this disease is neural at its inception, inasmuch as t spread of micro-organisms is either in or along the finest cutaneous ner fibres in the initial stages, probably gaining entrance through hair follicl The bacilli are then attracted towards the degenerating or regenerating ner fibres in the cutaneous plexus. Wyburn and Bacsich (1950) have shown t rapidity and ease with which hyaluronidase penetrates into nerve trunks a they also found that the effect of hyaluronidase used in this manner lasted

ırs and showed no ill after-effects. Any therapeutic measure designed to ıtrol the course of the disease would have to reach the axonal substance so :o be able to exert its influence on the bacilli. Our own observations on the : of hyaluronidase in rendering cutaneous nerve fibres and terminals access- e to staining or impregnating agents would seem to be of particular import- ce in this connexion.

This work was made possible by a grant from the Rockefeller Foundation ıich is gratefully acknowledged. We would also like to thank Miss Jean ırden and Mr. Frank Blackwell for their skilled technical assistance.

REFERENCES

ıRAN-REYNALS, F., 1929. 'The effect of certain organs from normal and immunised animals on the infecting power of vaccine virus.' J. exp. Med. **50**, 327.

INDEL, W. H., ALLISON, A. C., and WEDDELL, G., 1948. 'Intravenous methylene blue for experimental studies on the central nervous system.' J. Neurol. Neurosurg. and Psychiat. **11**, 227.

VING, E. A., and TOMLIN, S. G., 1954. 'Collagen, reticulum and their argyro philic properties.' Proc. Roy. Soc. B, **142**, 113.

HANOLKAR, V. R., 1951. 'Studies in the histology of early lesions in leprosy.' Indian Council med. Res., Special report No. 19.

CGREGOR, I. A., 1953. 'Hyaluronidase as an adjuvant to methylene blue in staining of nerve fibres.' Nature, **171**, 1070.

HMORI, D., 1924. 'Über die Entwicklung der Innervation der Genitalapparate als peripherer Aufnahmeapparat der genitalen Reflexe.' Z. ges. Anat. 1. Z. Anat. EntwGesch., **70**, 347.

ALLIE, W., CORNER, G. W., and WEDDELL, G., 1954. 'Nerve terminations in the myometrium of the rabbit.' Anat. Record (in the press).

OMANES, G. J., 1950. 'The staining of nerve fibres in paraffin sections with silver.' J. Anat. Lond., **84**, 104.

VEDDELL, G., 1941. 'The pattern of cutaneous innervation in relation to cutaneous sensibility.' Ibid., **75**, 346.

VEDDELL, G., and ZANDER, E., 1950. 'A critical evaluation of methods used to demonstrate tissue neural elements, illustrated by reference to the cornea.' Ibid., **84**, 168.

—— —— 1951. 'The fragility of non-myelinated nerve terminals.' Ibid., **85**, 242.

VYBURN, G. M., and BACSICH, P., 1950. 'Current therapeutics: hyaluronidase.' Practitioner, **164**, 361.

BY APPOINTMENT TO
H.M QUEEN ELIZABETH THE QUEEN MOTHER —— THE LATE KING GEORGE VI ——
SUPPLIERS OF PHOTOGRAPHIC EQUIPMENT

WALLACE HEATON LTD

NEW Research, Laboratory & Students MICROSCOPES BY PRIOR, BECK, WATSON, SWIFT, ETC.

Also a Large Selection of Second-hand Stands always in stock, guaranteed to be in first-class condition **from £10–£150**

REPAIRS, OVERHAULS, ETC.

We undertake in our own workshops by experienced scientific workers, repairs, overhauls, etc., to your own scientific equipment and turn itout practically like new. Estimates given on sight of instruments.

EASY PAYMENTS AVAILABLE

We are glad to announce that our easy payment plan is available for both new and secondhand equipment costing £10 or more. 25% deposit, the balance payable in 12 or 24 further monthly instalments. The plan is simple and straightforward. Please write for form.

127 NEW BOND STREET, LONDON, W.1

Phone: Mayfair 7511

Volume 95. Part 3

CONTENTS

NOTICE TO CONTRIBUTORS inside front cover

The Quarterly Journal of MICROSCOPICAL SCIENCE

THE SUBSCRIPTION for Vol. 95 is £4. 4s. 0d. for the Four Numbers; for this sum (*prep*
the JOURNAL is sent Post Free to any part of the world. Separate numbers and back num
which remain in print are sold at 30s. net each.

At the CLARENDON PRESS, OXFORD, *or*
OXFORD UNIVERSITY PRESS, LONDON, E.C. 4

Back numbers of the Second Series, Vols. 65–87, may be obtained from
THE SECRETARY, THE COMPANY OF BIOLOGISTS LTD.,
ZOOLOGICAL LABORATORY, DOWNING STREET,
CAMBRIDGE, ENGLAND.

PRINTED IN GREAT BRITAIN BY CHARLES BATEY AT THE UNIVERSITY PRESS, OXFORD